## MITIGATING THE DOWNSTREAM EFFECTS OF EXCESS SOIL PHOSPHORUS THROUGH CULTIVAR SELECTION AND INCREASED FOLIAR RESORPTION

by

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# A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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### ABSTRACT

Elemental phosphorus has been categorized as a non-renewable resource that is crucial to global food security. This is largely due to the transport of phosphorus being dependent on aqueous transfer and therefore an inherent inability to return to upstream ecosystems. It is only through mining and transport of rock phosphate that agricultural land remains productive. Simultaneously, due to agricultural over-fertilization, phosphorus has been characterized as a pollutant in aquatic environments. Diffuse source run-off from high phosphorus soils continues to contribute to downstream eutrophication decades after nutrient management practices have been put into place. A potential solution involves planting high biomass-producing tree species along riparian areas. Trees belonging to the Salicaceae are ideal candidates as they have a wide geographical distribution in Canada and broad-scale applications, ranging from fibre production to biofuel feedstock to uses in phytoremediation. The objective of this thesis was to identify a commercially available tree genotype, be it poplar or willow, well suited for widespread planting in agricultural areas to limit nutrient enrichment of riparian ecosystems. Phenotypic differences in phosphorus storage and allocation were analyzed using ICP-AES and HPLC. Poplar varieties Tristis and Northwest demonstrated the highest capacity for luxury uptake with an estimated 3.7 - 3.9 mg P g<sup>-1</sup> when 2.2 mM soluble phosphate (100N:70P) was applied, with no measurable metabolic consequences. However, the majority of phosphorus was stored in leaves as phosphate and subsequently returned to the environment as autumnal senescence progressed. This led to the exploration of factors limiting phosphate translocation and resorption. Expression of an exogenous phosphate  $H^+/H_2PO_4^-$  symporter in poplar led to a small, but significant increase in phosphate resorption and a pronounced increase in sulfate resorption, leading to further questions surrounding anion efflux from the vacuole and the role of the tonoplast in limiting nutrient translocation. If resorption proficiency could be increased under the high nutrient loads found in productive lands, poplar genotypes with luxury consumption could be bred for improved resorption and used to reduce phosphorus entry into riparian ecosystems. Extrapolation of this information to crop species could lead to reduced fertilizer application and improved nutrient management of perennial production systems.

# LAY SUMMARY

For the past 40 years, phosphorus has accumulated in several regions across the Canadian landscape, largely owing to its presence in fertilizers and detergents. As phosphorus levels in surface water runoff increase, algal blooms (eutrophication) manifest in downstream lakes, resulting in decreased water quality, aquatic species mortality and loss of recreational space. Given that phosphorus is prevalent throughout the environment, large-scale extraction is not an economically feasible option.

A practical solution may lie in current agroforestry practices. Commercially available hybrid poplar and willow trees are often planted on marginal lands surrounding agricultural fields to act as windbreaks and riparian buffers. Harnessing the ability of these trees for uptake, storage and remobilization of phosphorus in above-ground tissue could facilitate the long-term management of phosphorus levels in both water and soil, while providing a reliable source of biomass for fibre, biofuel production and even biochar.

## PREFACE

Of the research chapters included in this thesis, three were written with the intent of publishing in peer–reviewed journals. My own contributions include proposal development, experimental design, execution of experiments as well as collecting and analyzing the data for chapters 2, 3 and 4. For chapter 2, Raju Soolanayakanahally identified the research opportunity and helped establish the experimental design, Rob Guy assisted with the experimental design and data interpretation, and my entire lab group aided in sample collection. Undergraduate research assistants Simran Cheema and Meghan Bonnell helped with sample preparation and processing for chapter 3. Madeleine Wilson assisted with the execution, collection and interpretation of the Arabidopsis T-DNA data in chapter 4. My supervisor, Shawn Mansfield, was involved with identifying and designing the research program, data interpretation and editing of all chapters.

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To my family

## **1. INTRODUCTION**

## **1.1 HISTORY**

In the wake of the Industrial Revolution (1760–1850) and the Green Revolution (1940– 1970), uncontrolled discharge of sewage, trace metals, organic chemicals, industrial solvents and nutrient contaminants was a common occurrence across much of North America and Europe. Although many issues concerning public and environmental health, such as asbestos, exposure to benzene and polychlorinated biphenyls, depletion of the ozone layer, forest dieback and freshwater algal blooms were identified, addressing these issues proved difficult (Harremoes et al., 2002). Preliminary studies examined large amounts of ecological (systems) data that often-revealed an association, a link between issue and possible causes, which could easily be debated among the scientific community and policy makers. The lack of direct causation and desired 'completeness' of the conclusions often resulted in inaction leading to increasing social and environmental costs (Harremoes et al., 2002; Hill, 1965). It was not until the 1970s when the precautionary principle ('better safe than sorry') came into effect and policy makers began to implement pollution reduction and prevention policies such as the Federal Water Pollution Control Act (1972) in the United States, the German Clean Air Act (1974), and the Canada Water Act (1985), to mention only a few (Branch, 2014; Harremoes et al., 2002). These policies aimed to reduce the risk of serious or irreversible impacts before conclusive evidence was available. The results were the creation of governing bodies such as the Environmental Protection Agency (1970) in the United States, the Department of the Environment (1985) in Canada and the European Environment Agency (1990) in the European Union, all of which have the mandate to preserve and enhance environmental quality.

In order to improve the quality of the environment, contaminated sites and the risk they pose to human health or the environment had to be identified, assessed and steps towards remediation needed to be taken based on the contaminant(s) in question. In the 1990s, common approaches to treating contaminated soil *in situ* included soil vapour extraction, stabilization in soil, acid or solvent washing/leaching and thermal treatments (Salt et al., 1995; Sims, 1990). Bioremediation, or the use of microorganisms to degrade or detoxify hazardous material in the soil of contaminated sites, was also encouraged given that it provided a long–term, site–specific solution and was a welcome alternative to chemical treatments (Sims, 1990). In turn, it was the

1

innate limitations of bioremediation, such as its ineffectiveness at neutralizing contaminants that cannot undergo chemical degradation (heavy metals and radionuclides), its inability to prevent contaminant percolation and leaching through soil horizons and the high soil carbon requirements of the microorganisms, that gave rise to use of vegetation in the treatment of contaminated soils (Salt et al., 1995; Schnoor et al., 1995).

## **1.2 Phytoremediation**

Phytoremediation is a general term indicating the use of plants to remediate environmental sites that have been contaminated by discharge from urban, industrial or agricultural sources. Pollutants can include organic contaminants, trace metals and excess nutrients, such as nitrates and phosphates (Salt et al., 1995; Schnoor et al., 1995).

#### **1.2.1 ORGANIC CONTAMINANTS**

Organic pollutants encompass a broad range of environmentally persistent chemicals that have generally been produced for industrial purposes (solvents, cooling fluids, construction, etc.) or for use in agriculture as pesticides (Jones and de Voogt, 1999). Plant removal of organic contaminants can be estimated by comparing a compound's solubility in octanol to its solubility in water. This ratio is called the octanol–water partition coefficient ( $K_{ow}$ ), and log  $K_{ow}$  is used to assess the level of soil adsorption that can be expected for any given organic compound. Organic contaminants with intermediate polarity such as benzene, toluene and various chlorinated solvents (log  $K_{ow} = 0.5 - 3$ ) are efficiently and passively transported to the shoots of plants, where they may be degraded or volatilize. Highly hydrophobic compounds with a log  $K_{ow} > 3.0$ , such as polychlorinated biphenyls (PCBs), bio–accumulate in the roots where they are thought to be bound to the cell wall, while compounds with a log  $K_{ow} < 0.5$  are not easily translocated due to their lack of interaction within the root zone (Ryan et al., 1988; Schnoor et al., 1995).

Although the translocation efficiency of organic contaminants with intermediate polarity was initially determined in a lab setting using barley (Briggs et al., 1982; Topp et al., 1986) and cress (Topp et al., 1986), field observations of the evapotranspiration of industrial organic solvents such as trichloroethylene (TCE) via English oak (*Quercus robur*) and common ash (*Fraxinus excelsior*) on a TCE-contaminated site have also been reported (Weyens et al., 2009). Degradation of the translocated contaminants is highly dependent on the contaminant in question and the

species selected for phytoremediation. Plant enzymes such as nitroreductase and laccase have been successful in degrading trinitrotoluene (TNT) in field crop trials, while dehalogenase aids in reducing TCE to chloride, carbon dioxide and water (Chekol et al., 2002; Schnoor et al., 1995; Weyens et al., 2009). The additional decontamination capacity of the microbial community supported by plant root exudates should not be discounted.

#### **1.2.2 TRACE METALS**

Metals are of environmental concern, as toxic effects in both plants and animals occur at relatively low concentrations, compared to other elements required for life. A variety of metals are introduced anthropogenically via mine tailings, pesticides, biosolids and other residues, including: As, Cd, Cr, Cu, Pb, Hg, Ni, Se, Mo, Zn, Tl, and Sb (Vamerali et al., 2010). At a basic level, the establishment of a vegetative cover on mine sites is beneficial, as it improves soil quality and may reduce metal leaching (Arienzo et al., 2004; Salt et al., 1995); however, identifying plants that display a defined level of metal removal from soil into aerial tissues is preferable. This initiated research into identifying plant species that accumulate above-normal levels of metals in their above-ground biomass. As a result, more than 400 metal-accumulating taxa have been found (Vamerali et al., 2010; Vassilev et al., 2004). These hyperaccumulators tolerate high metal soil conditions due to their increased ability to sequester metal ions in the vacuole, by using phytochelatins or metallothioneins to bind metal cations or by metal complexation with histidine (Gratão et al., 2005; Salt et al., 1995; Seregin and Kozhevnikova, 2008). High biomass-producing tree species such as poplar (Populus spp.) and willow (Salix spp.) are also considered candidates for metal phytoextraction. Although they are not hyperaccumulators, interclonal variation and biomass production can lead to a similar Cd extraction potential on a yearly basis, while providing many phytostabilization benefits, such as soil stabilization and reduction of metal leaching (Laureysens et al., 2004; Pulford and Watson, 2003; Vassilev et al., 2004).

#### **1.2.3** NUTRIENT ENRICHMENT

Nitrogen and phosphorus are the two nutrients of primary concern when addressing the issue of nutrient enrichment in aquatic systems (Howarth et al., 1996; Sansom and Kinser, 2012). Major sources of these elements include agricultural runoff, urban and industrial effluents, urban runoff, recreational land use and atmospheric deposition; each source can be classified as point or non–point source pollution. Point source pollution, such as discharge of municipal waste, has been

regulated since the 1970s with municipalities in Eastern Canada and the U.S. initiating secondary treatment of effluent in order to minimize the release of nitrates, ammonium and phosphates (Watkinson and Poston, 1968). Non-point sources have no distinct location at which they enter waterways, making it difficult to measure and control pollutant levels (Ongley, 1996; Seitzinger et al., 2005). In 1994, the United States Environmental Protection Agency (US-EPA) found that 72 % of river length and 56 % of lakes in assessed areas were impacted by agriculture, leading to the declaration that agriculture was the main source of water quality impairment in the United States. Equally, in 1996 the United Nations Environment Programme discovered that agriculture was the primary source of phosphorus in the coastal zone of Mediterranean countries (Ongley, 1996). For phosphorus in particular, two decades of attempts to reduce non-point source pollution through improved land management have yielded little in terms of algal bloom prevention and improved water quality in freshwater catchments (Jarvie et al., 2013). Terrestrial losses of phosphorous are an artefact of past land use and stem from the release of dissolved phosphorus from sediment as the soil phosphorus storage capacity nears saturation. Consequently, it has been estimated that it will take decades to deplete these soil phosphorus reserves on land where best management practices are applied (Jarvie et al., 2013). In order to address the issue of non-point source nutrient enrichment, buffer strips of high-biomass-producing tree species can be planted along riparian zones to stabilize river banks, while concurrently filtering nutrients from water runoff (Fillion et al., 2011; Kneteman, 2012).

#### **1.2.4** LIMITATIONS

Phytoremediation is a promising technology, capable of both terrestrial and aquatic remediation, but it is not without its limitations. On highly contaminated sites, plant establishment may be impeded by overly high levels of toxicity, while less contaminated soils may not contain the macronutrients, soil-water-holding capacity and/or pH required for growth (Pulford and Watson, 2003). Estimated remediation times of high-performing plant species are generally long, varying between 5 and 25 years, depending on soil type and the bioavailability of the contaminant over time (Pulford and Watson, 2003; Salt et al., 1998; Vamerali et al., 2010). Careful site-specific selection of high-biomass-producing species tolerant to the mix of environmental contaminants must also be performed, which is both a limitation and advantage of the technology. Although the degradation or removal of contaminants via phytoremediation is effective only when dealing with moderately hydrophobic chemicals or excess nutrients found at a depth no greater than 5 m, the

benefits of phytostabilization achieved through the establishment of vegetation are crucial to the long-term management and remediation of any contaminated site (Schnoor et al., 1995).

## **1.3 Phosphorus**

Phosphorus, in the form of phosphate esters, permeates throughout biological systems due to its dual roles as a major component of genetic material and various metabolic intermediates, while also acting as a source of energy (Westheimer, 1987). In the absence of enzymes, phosphate ester anions have a low rate of hydrolysis, resulting in stable intermediary compounds that are maintained until the addition of the required enzymes, thereby allowing for controlled reactions to occur within the cell. Although phosphorus is essential to life, it makes up only 0.09 % of the Earth's crust (by weight), and is therefore considered a limiting nutrient for the growth of primary producers in the majority of ecosystems (Elwood et al., 1981; Filippelli, 2008; Oelkers and Valsami-Jones, 2008). Over the course of the past 30 years, phosphorus has also become a contaminant of concern due to its indirect effect on water quality via eutrophication, resulting in the establishment of regional environmental quality guidelines for phosphorus (Government of Canada, 2004). Adherence to these guidelines relies heavily on our ability to reduce anthropogenic inputs of phosphorus, while concomitantly mitigating the long–term effects of phosphorus release from soils already saturated with phosphorus (Jarvie et al., 2013).

## **1.3.1** PHOSPHORUS CYCLE

Phosphorus exists naturally in a single environmentally stable form as phosphate. Present as either an inorganic salt or ester, phosphate does not form a stable gas phase unlike other essential elements, such as nitrogen and carbon. This causes the transport of phosphorus to be almost exclusively dependent on aqueous transfer (Emsley, 1980; Filippelli, 2008). Furthermore, only a few minerals, the calcium phosphates known collectively as apatites, contain phosphorus in appreciable amounts, and only one form of calcium phosphate is soluble in water (Table 1.1). Once calcium phosphates precipitate out of solution, they slowly revert to hydroxyapatite or fluoroapatite, which accumulate to form large phosphate rock deposits (Emsley, 1980).

		Ratio Ca: P	Solubility in Water
Calcium dihydrogen phosphate hydrate	$Ca(H_2PO_4)_2 \cdot H_2O$	0.5	Soluble
Calcium hydrogen phosphate dihydrate	$CaHPO_4\cdot 2H_2O$	1.0	Insoluble
Octacalcium phosphate pentahydrate	$Ca_8H_2(PO_4)_6\cdot 5H_2O$	1.33	Insoluble
Calcium phosphate	Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	1.5	Insoluble
Hydroxyapatite	Ca <sub>5</sub> (PO <sub>4</sub> ) <sub>3</sub> OH	1.67	Insoluble
Fluoroapatite	$Ca_5(PO_4)_3F$	1.67	Insoluble

 TABLE 1.1: LIST OF CALCIUM PHOSPHATES; TABLE ADAPTED FROM EMSLEY 1980.

Weathering of phosphate from igneous or sedimentary rock occurs when dissolved carbon dioxide (as carbonic acid) leads to the dissolution of apatite minerals. Formation of carbon dioxide also occurs biochemically in soils through bacterial respiration which causes the acidification of the surrounding soil. Similarly, exudates from bacteria and plant roots, consisting of organic ligands in the form of citrate and oxalate, can dissolve apatite via acidification and the chelation of calcium, thereby increasing the concentrations of  $H_2PO_4^-$  in the soil solution (Bucher, 2007; Cunningham and Kuiack, 1992). Once in solution, the phosphate is immediately bioavailable and acquired by plants and other primary producers (Filippelli, 2008). In plant tissues, phosphate is converted to organic phosphate in the form of phospholipids, nucleic acids and inositol phosphates. After plant senescence and tissue death, the organic phosphate esters are found in the soil primarily as inositol phosphates ( $\leq 50$  %) with a small fraction being comprised of nucleotides ( $\leq 3$  %) and phospholipids (0.5 - 7.0 %). The remainder includes traces of phosphonates and the remaining unidentified fraction (Brady, 1977; Stewart and Tiessen, 1987; Turner et al., 2003). Given that phospholipids and nucleic acids are initially of greater abundance than inositol phosphate in plant material, it is assumed that they are rapidly mineralized by bacteria and therefore do not persist in the soil matrix. Fungal and plant exudates containing phosphatases also contribute to the mineralization of organic phosphates so that inorganic phosphate is once again available in the soil solution (Bucher, 2007; Cosgrove et al., 1970).

The withdrawal of phosphate from the soil solution has varying effects on its accessibility. Forms of phosphate that remain relatively accessible are termed labile or unstable. These forms include dissolved phosphate, organic phosphates and phosphate that has been adsorbed onto positively charged soil particles, primarily those containing aluminum hydroxide. Occluded or stable forms of phosphate comprise those that have precipitated as Ca, Fe, Al and Mn complexes. In neutral and alkaline soils, phosphate preferentially precipitates as calcium phosphates, while Al and Fe precipitates dominate in acidic conditions, where soil pH is between 3.0 - 6.0 (Chang and Chu, 1961; Emsley, 1980; Hinsinger, 2001). In the majority of soils, the formation of the less soluble iron phosphate complex is favoured and the rate of formation is positively correlated to soil moisture (Chang and Chu, 1961). As dissolved phosphate is removed from the soil solution, a zone of depletion is created around plant roots, leading to the formation of a concentration gradient that favours de-absorption and dissolution of stable phosphate. Unfortunately, the release of phosphate from stable sources via diffusion occurs too slowly to maintain solution concentrations high enough to support rapid plant growth (Schachtman et al., 1998). To counter this effect on agriculturally suitable lands, soluble phosphate is added to maintain high phosphate concentrations in solution where it is inexorably fixed in most soil types in as little as three hours (Chang and Chu, 1961). Should anthropogenic inputs cease, over time total available phosphorus would be lost through surface and subsurface runoff until a steady-state equilibrium is achieved and the ecosystem becomes heavily reliant on phosphorus that is recycled within the ecosystem (Filippelli, 2008; McDowell and Sharpley, 2001).

In terms of phosphate transfer between ecosystems, rivers act as the primary method of transport. In river systems, phosphorus exists in three main forms: inorganic phosphate, described as dissolved reactive phosphorus (DRP); soil-adsorbed phosphorus, described as total particulate phosphorus (TPP); and dissolved organic phosphorus (DOP). The sum of the three forms is referred to as total phosphorus (TP), and levels within streams are governmentally regulated based on TP values, due to the variance associated with measuring biologically active levels of phosphorus in phosphorus-limited ecosystems (Hudson et al., 2000). As rainwater or freshet travels through soil, it accumulates both inorganic and organic phosphorus, transporting it along surface or subsurface channels into river systems. This results in phosphorus being naturally introduced from forested ecosystems into rivers and streams, predominately in particulate form as soil and leaf litter, but in relatively small amounts (< 0.1 kg ha<sup>-1</sup>yr<sup>-1</sup>) (Wetzel, 2001; Withers and Jarvie, 2008). Phosphorus loading of tributaries and water catchments undergoes seasonal fluctuation, with higher loading and increased sedimentation occurring for much of the year from autumn to spring (Kennedy et al., 1986). Anthropogenic inputs are highly variable in terms of

source (point or diffuse), chemical composition (DRP, TPP or DOP), as well as concentration, with reported TP input estimates in the U.K. ranging from 0.02 mg L<sup>-1</sup> to 247 mg L<sup>-1</sup> (Withers and Jarvie, 2008). Worldwide anthropogenic DRP inputs are estimated to range between 0.05 - 1 mg L<sup>-1</sup> compared to the natural baseline of 0.01 mg L<sup>-1</sup> (Meybeck, 1982).

Once particulate phosphorus accumulates in natural water catchments, the anoxic conditions, generated in lakes during the summer months by primary producers, favour the reductive dissolution of Fe phosphates from the suspended sediment (House, 2003). This occurs once the redox potential reaches +200 mV (Wetzel, 2001). The resulting release of inorganic phosphate is capable of maintaining high internal phosphorus concentrations, even during periods of reduced external loading (Government of Canada, 2004). The effect on lake water quality and ecosystems has been found to be largely dependent on the volume-to-surface ratio of the lake in question (Salomons et al., 1987).

The final step in the phosphorus cycle is the eventual deposition of phosphorus–bearing sediment into the marine environment. Phosphorus concentrations in the ocean differ with depth. Surface depletion is caused by phosphorus uptake by phytoplankton, and therefore concentrations gradually increase as light becomes limiting (Filippelli, 2008). Given that phosphorus cannot return to upstream ecosystems, it is considered to be a non–renewable resource, and the phosphorus cycle is often referred to as 'the phosphate spiral'. It is only through the mining and artificial transport of phosphorus–ladened mineral deposits that agricultural land is able to continually support high crop yields while removing the majority of plant biomass. With projected increase in demand for food, fuel, and fibre, it is suggested that half of the world's current phosphate resources (economically viable deposits containing 20 - 30 % P<sub>2</sub>O<sub>5</sub>) will be consumed within the next 60 years for use as fertilizer. Limiting the export of phosphorus out of areas of high productivity is therefore essential to prevent eutrophication and required for the conservation of global phosphate reserves (Elser and Bennett, 2011; Oelkers and Valsami-Jones, 2008).

#### **1.3.2 PHOSPHATE AS A WATER POLLUTANT**

Phosphorus levels in surface drainage are a product of soil phosphorus, topography, vegetative cover, volume of overland flow and land use, all of which contribute to the variable levels of phosphorus loading in lakes that are largely dependent on the drainage basin in question. Uncontaminated freshwaters contain between 0.01 - 0.05 mg L<sup>-1</sup> of TP (Wetzel, 2001) and are

considered to be phosphorus limited based on the rapid uptake constants observed upon addition of phosphate (Hudson et al., 2000). Phosphorus has been targeted as an element of concern in circumstances of nutrient enrichment due to its primary role in stimulating algal growth in freshwater catchments. This was most acutely demonstrated in experiments performed at the Experimental Lakes Area (ELA) in Ontario, Canada, where the established Redfield ratio, the 40:7:1 mass ratio of carbon:nitrogen:phosphorus proven to be optimal for the growth of marine phytoplankton (Redfield, 1958), was tested.

In the first experiment, Lake 226 was divided into two halves where the southwest portion was fertilized with sucrose and sodium nitrate (10C:5N) while the northeast segment was fertilized with sucrose, sodium nitrate and phosphoric acid (10C:5N:1P). The resulting phytoplankton blooms, dominated by nitrogen-fixing cyanobacteria in the northeast basin, and the lack thereof in the southwest basin, visibly demonstrated the importance of phosphorus as an aquatic pollutant in natural ecosystems (Findlay and Kasian, 1987). However, the biomass increase of 2 - 4xobserved in the southwest basin (fertilized with C and N only) when compared to the unfertilized control lakes was a cause for debate among the scientific community, as it was considered as further support for the hypothesis that nitrogen was the limiting nutrient for eutrophication (Howarth and Marino, 2006). The C, N, P fertilized northeast basin had 4 - 8x more biomass than the unfertilized controls (Findlay and Kasian, 1987). To dispute the theory that international policy should focus on reducing nitrogen inputs to counteract eutrophication, data from a second nutrient enrichment experiment was brought forward. Lake 227 in the ELA was fertilized on an annual basis with phosphoric acid and sodium nitrate at a ratio of 12:1 (by weight) for five years. For the following 15 years, fertilizer was added at a ratio below the Redfield ratio (4N:1P), with an observed effect on the distribution of species within the phytoplankton community. Over the next 17 years, phosphorus was continually added without the addition of nitrogen. Throughout the course of the 37-year experiment, there were no significant differences in the annual averages of phytoplankton biomass. At no point did the N:P ratio within the lake drop below the Redfield ratio of 7:1, and the lake maintained an average TP of  $0.042 \text{ mg L}^{-1}$ . Final conclusions suggest that the lake was able to become carbon and nitrogen sufficient over time due to atmospheric fixation and subsequent recycling of these elements; suggesting the futility of attempting to control either element for the purpose of reducing the effects of eutrophication (Schindler et al., 2008). Therefore, while available nitrogen affects phytoplankton species composition, biomass production and algal

bloom severity are determined by the availability of phosphorus in freshwater systems. A similar conclusion could be reached for marine ecosystems; however, metal concentrations and salinity may hinder the growth of N–fixing cyanobacteria, meaning that the addition of nitrogen may be required for an algal bloom to occur (Boesch et al., 2006; Schindler et al., 2008). Due to its lack of mobility between ecosystems, phosphorus is often the most limiting nutrient for growth and is primarily responsible for seasonal algal blooms.

This research paved the way for policymakers to focus primarily on reducing anthropogenic phosphorus inputs and economically reduce the eutrophic symptoms of high turbidity, anoxia and algal blooms in freshwater ecosystems. This involved sewage treatment in many European countries and North America to varying levels of success. Point source sewage treatment is not always sufficient; as demonstrated by the River Thames in the U.K., where the released treated water still contains enough phosphorus to cause DRP concentrations in the river to remain well above 0.5 mg P  $L^{-1}$  during low flow periods (Jarvie et al., 2006). In less densely populated regions, such as Sweden, the implementation of sewage treatment plants in the 1970s had greater success with treatments reducing outgoing sewage N:P ratios from 7N:1P to 26N:1P prior to its discharge into the Swedish archipelago. TP concentrations within the archipelago did not show a marked decline until the 1990s, suggesting a lengthy dissipation time for internal phosphorus loads (Boesch et al., 2006). Concurrent sewage treatment in Canada and the United States, in an effort to reduce pollutant levels in the Great Lakes, has also led to stabilized or decreasing trends in phosphorus levels in all five lakes (1970 - 2010) (Environment Canada, 2013; International Joint Commission, 2014). Regrettably, even with decreasing trends in phosphorus levels, algal blooms still continue to affect both the Baltic Sea and the Great Lake regions, indicative of the importance of controlling non-point source nutrient pollution through land management (Boesch et al., 2006; International Joint Commission, 2014; Andersen et al., 2017). This predominantly refers to agricultural practices given that 50 % of annual phosphorus loads in the U.K. have been determined to come from agricultural diffuse sources, and agriculture has been determined to be the primary cause of reduced water quality in the United States and in areas surrounding the Mediterranean Sea (Ongley, 1996; Jarvie et al., 2006; Spiteri et al., 2016). Even with water treatment legislation in effect, stream monitoring in the United States demonstrated that out of 250 river sites, 75 % had total phosphorus concentrations of 0.46 mg  $L^{-1}$  or less with a median value of 0.12 mg  $L^{-1}$  (biological limitation of algae production is 0.03 mgTP  $L^{-1}$ )

(Hamilton, 2012). Agricultural diffuse sources include runoff from intensive animal-husbandry operations, fertilization of crops with soluble inorganic phosphate, and erosion as a result of deforestation and tillage (International Joint Commission, 2014; Manitoba Phosphorus Expert Committee, 2006; Ongley, 1996). These types of anthropogenic inputs are the most difficult to reduce and regulate due to the large land base within most agricultural watersheds, affecting the practicality of both targeted reduction and reinforcement of best management practices.

Agriculture is the largest contributor to soil phosphorus on an annual basis, yet urban pointsource effluents can result in high local concentrations of phosphorus, which in turn provides an ideal environment for algal growth, particularly during low-flow periods (Jarvie et al., 2006). This complexity further confounds attempts to legislate reductions in anthropogenic phosphorus loading and has inhibited the establishment of phosphorus environmental quality guidelines at a national level. Only the Netherlands and Australasia have national water-quality standards of 0.15 mgTP  $L^{-1}$  and 0.035 - 0.050 mgTP  $L^{-1}$ , respectively. In other countries, such as Canada, provincial guidelines are a common practice. With the exception of Atlantic Canada, all other provinces have set provincial phosphorus guidelines ranging between the lowest in British Columbia (0.005 –  $0.015 \text{ mgTP } \text{L}^{-1}$ ) to the highest allowable level of  $0.050 \text{ mgTP } \text{L}^{-1}$  in Alberta. Only three states in the United States have set guidelines, ranging from 0.005 - 0.025 mgTP L<sup>-1</sup>. The European Economic Community member states have taken an alternative approach and most countries aim to reduce phosphorus inputs rather than set definitive water quality standards (Government of Canada, 2004). To impact further anthropogenic loading of waterways and have the potential to reach the abovementioned guidelines, further legislative incentives are required. With the knowledge that the control of point-source pollution is not sufficient for the reduction of phosphorus levels, whole system ecological approaches are required to find multiple, economically viable, site-dependent solutions capable of safeguarding the multiple entry points available to nonpoint source pollutants. Phytoremediation has the potential to meet such requirements and become one of the many solutions, should appropriate species be identified for use in Canada.

## **1.4 PHOSPHORUS IN PLANTS**

Phosphorus is found in concentrations ranging from 0.1 - 1 % of dry matter in plant tissues, making it one of three most abundant nutrients, alongside nitrogen and potassium (Hernández and Munné-Bosch, 2015). Its inorganic form, known as phosphate (Pi), is one of the most important ions in plant nutrition, with roles in the majority of developmental and regulatory processes ranging from energy metabolism to the formation of membrane phospholipids. To ensure proper metabolic function and signalling, constant levels of available Pi are maintained in the cytosol via vacuolar reservoirs. Under phosphorus sufficient conditions, between 85 - 95 % of total Pi is stored within plant cell vacuoles (Marschner, 2012). This separation of the two inorganic pools, a storage Pi pool in the vacuole and a metabolic Pi pool in the cytosol, chloroplasts and mitochondria, allows levels of photosynthesis and respiration to be maintained when plants are exposed to an overabundance or insufficient quantity of Pi (Mimura et al., 1990).

### **1.4.1 TRANSPORT AND ACCUMULATION**

Plants are predominantly exposed to conditions of Pi deficiency due to limiting amounts of bioavailable phosphorus in the soil. The nutrient status of plants is therefore greatly affected by environmental concentrations. From the soil solution, phosphate is actively taken up by root cortical cells and travels through the symplast to the xylem. Within the xylem, parenchyma cells control phosphorus loading and ion levels into the xylem fluid, maintaining Pi levels at about 2 - 5 % of the levels found in roots. Phosphate then travels through the apoplast to the aerial sink tissues, such as actively growing meristems and young leaves (Bieleski and Ferguson, 1983; Poirier et al., 1991). As concentrations in the external environment increase, xylem apoplastic levels of Pi have been shown to remain relatively stable. Concurrent accumulation of phosphate in leaves as external concentrations increase suggests that leaf mesophyll cells have a large capacity for Pi import and compartmentalization when exposed to excess Pi conditions (Mimura et al., 1990). Optimal total phosphorus concentrations in crop photosynthetic tissues are less than 4 mg P g<sup>-1</sup>, with concentrations reaching above 5 mg P g<sup>-1</sup> in tissues of plants growing in high phosphate conditions (Veneklaas et al., 2012).

Specifically in leaf cells, due to the high demand for phosphorylated intermediates, photosynthesis and carbon partitioning can be significantly affected by cytosolic Pi concentrations as they affect plastid phosphate levels. Optimal Pi concentrations within the chloroplasts generally

equilibrate between 2.0 - 2.5 mM and can fall below 1.4 - 1.0 mM during times of Pi deficiency, leading to the near complete inhibition of photosynthesis. Increases in Pi, of up to 20x initial concentrations, do not appear to hinder photosynthesis but alter carbon allocation between starch and sucrose. Subjecting isolated chloroplasts to external Pi concentrations of up to 1 mM led to the inhibition of starch synthesis via the allosteric hindrance of the key enzyme required for starch synthesis, ADP-glucose pyrophosphorylase. Higher concentrations (5 mM) of Pi in the stroma resulted in near complete inhibition of starch synthesis. Concurrent excessive export of triosephosphates into the cytosol may further prevent starch synthesis due to substrate depletion, while limiting the ability of the chloroplasts to regenerate ribulose bisphosphate (RuBP) (Marschner, 2012). Phosphate homeostasis in excess conditions in plants is therefore largely dependent on the storage capacity of the vacuole. High cytosolic Pi concentrations reduce Pi uptake into the vacuole, suggesting the utility of vacuoles as efficient Pi sinks is determined by the established electrochemical gradient (Mimura et al., 1990). Within the vacuole, phosphate can also be stored in other forms such as precipitated polyphosphates (polyphosphates [( $PO_{3-}$ )n] with n = ~200) or inositol hexaphosphate (phytic acid), which is the chief form of organic phosphate storage that can be used to directly phosphorylate ADP (Emsley, 1980). As storage rates slow, mobilization and net export of phosphate from plant roots back into the environment then becomes essential to avoiding phosphorus-induced zinc and copper deficiencies, and can effectively compensate for higher Pi influx (Raghothama and Karthikeyan, 2005; Teng and Timmer, 1990).

Phosphorus accumulation in leaves has been observed to occur in many different species such as barley (*Hordeum vulgare*) (Mimura et al., 1990); rice (*Oryza sativa*) (Wang et al., 2014); poplar (*Populus* spp.) (Teng and Timmer, 1990); willow (*Salix* spp.) (Fillion et al., 2011) and many others (Loneragan and Asher, 1967) when exposed to nutrient solutions containing high levels of phosphorus. The same is true of plants adapted to phosphorus impoverished soils; however, unlike species adapted to moderate phosphorus levels, they are unable to reduce Pi uptake as internal concentrations increase. The ability to moderate Pi uptake has been shown to be negatively correlated with reabsorption efficiency in plants adapted to phosphorus impoverished environments (de Campos et al., 2013), which illustrates the importance of constitutively expressed phosphate transporters and their role in phosphorus entry into waterways.



FIGURE 1.1: DATA COMPILED FROM MULTIPLE SOURCES DEMONSTRATING THE FLUXES OF PHOSPHORUS POOLS IN CROP PHOTOSYNTHETIC TISSUE AS LEVELS OF PHOSPHORUS SUPPLY INCREASE (VENEKLASS ET AL. 2012). REPRINTED WITH PERMISSION OF JOHN WILEY & SONS INC.

#### **1.4.2 PHOSPHATE ACQUISITION**

Phosphorus is taken up by plants in the orthophosphate forms,  $H_2PO_4^-$  and  $HPO_4^{-2}$ , which occur at low concentrations in soil solutions  $(0.1 - 10 \,\mu\text{M})$ . Of these, the monovalent anion is preferentially transported, with optimal uptake occurring at a pH of 4.5–5.0 (Vance et al., 2003). Soil concentrations of Pi are typically several orders of magnitude lower than the 5 - 20 mM of phosphate found within plant cells (Raghothama, 1999), suggesting that uptake is energy dependent. This is further demonstrated by the sensitivity of the system to temperature and metabolic inhibitors. Phosphate uptake kinetics have demonstrated the synchronous activity of two systems; an inducible high-affinity ( $K_m 3 - 10 \mu M$ ) and a constitutive low-affinity system ( $K_m 5 - 10 \mu M$ ) 300 µM) (Bieleski and Ferguson, 1983; Raghothama and Karthikeyan, 2005), and characterized Arabidopsis transporters have been grouped into five phosphate transporter (Pht) families: Pht1, Pht2, Pht3, Pht4 and Pht5. The Pht1 gene family is thought to encode high-affinity H<sup>+</sup>/H<sub>2</sub>PO<sub>4</sub><sup>-</sup> symporters, located in the plasma membrane, that are expressed in root cells and are induced during Pi-deprivation (Lin et al., 2009). Subsequent reporter gene studies have expanded their role to include between-tissue Pi loading (Mudge et al., 2002). Pht2 consists of low-affinity transporters located in the chloroplast envelope whose mis-regulation affects Pi allocation within the plant (Versaw and Harrison, 2002), most likely due to inhibitory effects on photosynthesis.

Pht3 and Pht4 have been shown to be localized to the mitochondrial inner membrane and Golgi compartment, respectively (López-Arredondo et al., 2014). Pht5 is a recently described phosphate transporter family with three members, all located on the tonoplast. AtPht5–1 (Liu et al., 2016) or vacuolar phosphate transporter 1 (VPT1) is the most characterized member and has been shown to function as an ion channel that mediates vacuolar influx of anions including phosphate, sulfate, nitrate, chloride and malate, with phosphate as the preferred anion. Disruption in Pht5–1 results in plant hypersensitivity to both low–Pi and high–Pi conditions and overexpression leads to phosphate overaccumulation (Liu et al., 2015). Additional proteins involved with ion transport and Pi loading have been discovered using forward-genetic approaches and may lead to the addition of new Pht families (Hamburger et al., 2002; Liu et al., 2012). Given that the Pht1 gene family is the most well characterized group of Pi transporters, consisting of an array of high- and low-affinity transporters situated in the plasma membrane whose functions range from uptake to reallocation, it was chosen as the family of interest with regards to the phytoremediation of phosphorus-rich soils.

#### **1.4.3 PHT1 GENE FAMILY IN ARABIDOPSIS**

The Arabidopsis genome contains a total of nine members of the Pht1 family whose nomenclature has been established based on the guidelines of the Commission for Plant Gene Nomenclature (Mudge et al., 2002). Two genes of the Pht1 family (Pht1-1 and Pht1-4) have been demonstrated to function as Pi transporters. Promoter-reporter gene fusions of all nine genes have been generated and spatial expression patterns of these genes are well documented. Transporters 1–1 to 1–4 are expressed in the root epidermis, and are induced under Pi deprivation with Pht1–2 and Pht1-3 being the most upregulated (Mudge et al., 2002). Pht1-1 and Pht1-4, while contributing to Pi acquisition during deficiencies, are crucial for uptake in high-Pi conditions above 0.2 mM, suggesting that these transporters could be dual-affinity transporters (Shin et al., 2004). Pht1-5 expression was found primarily in the shoot, where it is expressed in the vasculature of cotyledons and is absent from leaves until the onset of senescence. During senescence, Pht1–5 is expressed in the leaf vasculature with the strongest expression in the phloem. Further study has implicated Pht1–5 in plant mobilization and reallocation of Pi from source to sink tissues (e.g. mature leaves to young leaves and mature leaves to roots) throughout growth (Nagarajan et al., 2011). The remaining Pht members are expressed in anthers and pollen grains (Pht1-6 and Pht1-7) (Mudge et al., 2002), or are root high-affinity transporters involved in Pi scavenging from the external environment during prolonged periods of deficiency (Pht1–8 and Pht1–9) (Remy et al., 2012). The redundant function and spatial expression of the Pht1 family have meant that mutations in a single Pht gene result in few physiological changes. Double to quadruple mutants of Pht1–1 to 1–4 have been observed to yield a significant phenotype. In these mutants, observed dual K<sub>m</sub> values of  $6.7\mu$ M and  $424\mu$ M were not affected yet V<sub>max</sub> was reduced, lending further support for the dual function of Pht1 gene family as low- and high-affinity transporters (Ayadi et al., 2015).

## **1.4.4 PHT1** GENE FAMILY IN OTHER SPECIES

Several members of the Pht1 gene family have been characterized in various crop species with the intent of finding candidate genes for improving phosphorus-use efficiency (PUE) and phosphate acquisition. The crop species involved include the following: rice (*Oryza sativa*), barley (*Hordeum vulgare*), soybean (*Glycine max*), tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). A commonality among these species is that all have Pht1 transporters with overlapping expression patterns and redundant function, emphasizing the importance of mineral nutrition in plants (Chen et al., 2014; Qin et al., 2012) (Table 1.2). Of particular interest for phytoremediation applications are low-affinity transporters involved in both the uptake and remobilization of phosphate; their constitutive expression has been implicated in the high phosphorus resorption efficiencies and inability to reduce phosphate uptake observed in plants growing in phosphorus deficient-environments (de Campos et al., 2013).

	P	ht1 gene family (			
Organism	Uptake	Translocation	Uptake & translocation	Mycorrhizal symbiosis	References
Arabidopsis	1-2, 1-3, 1-8, 1-9	1–5	1-1, 1-4	_	(Ayadi et al., 2015; Mudge et al., 2002; Nagarajan et al., 2011; Remy et al., 2012)
Rice	1-2, 1-3, 1-7	_	1-6, 1-8	1–11, 1–12, 1–13	(Ai et al., 2009; Glassop et al., 2007; Jia et al., 2011; Veneklaas et al., 2012)
Barley	1-1, 1-2, 1-3, 1-4	1–6	_	1–8	(Huang et al., 2011; Rae et al., 2003; Schünmann et al., 2004)
Soybean	1-3, 1-4, 1-12	1–10, 1–14	1–1, 1–2, 1–6, 1–7, 1–8, 1– 9, 1–11, 1–13	1–7	(Inoue et al., 2014; Qin et al., 2012; Wu et al., 2011)
Tomato	1-2, 1-3 1-6	_	1-1, 1-3, 1-7	1-3, 1-4, 1-5	(Chen et al., 2014; Liu et al., 1998)
Potato	1–2	_	1–1	1–3	(Leggewie et al., 1997; Rausch et al., 2001)

#### TABLE 1.2: LITERARY SUMMARY OF PUTATIVE PHT1 TRANSPORTER FUNCTIONS

#### 1.4.5 PHT1 GENE FAMILY IN POPLAR AND WILLOW

The assembled genome of *Populus trichocarpa* has led to the identification of 12 Pi transporters in the Pht1 gene family, whose expression levels vary with Pi availability and poplar genotype. Expression of the majority of Pi transporters is ubiquitous in adventitious roots, the central cylinder, the petiole and the mature leaves, excluding PtPht1–8 and 1–10, which are mycorrhiza–inducible. Transcripts of PtPht1–4 and PtPht1–7 could not be distinguished from one another due to their high sequence similarity and are found in the highest concentrations in all tissues (Loth-Pereda et al., 2011).

Since excess Pi accumulates in leaf tissue, many transporters could be considered candidates for gene mis-regulation, given that PtPht1-1, 1-2, 1-3, 1-5, 1-6, 1-9 and 1-12 are all similarly expressed in mature leaves (Loth-Pereda et al., 2011). PtPht1-1, 1-5, 1-6, 1-9 and 1-12 are also substantially upregulated during senescence. Since PtPht1-9 and 1-11 are likely to be recent genome duplications and show high sequence homology to the AtPht1-8 and 1-9 highaffinity transporters, they are more likely to be involved in Pi scavenging from the soil. Of the remaining transporters, PtPht 1-1 bears highest sequence homology to AtPht 1-1, which has confirmed function in both Pi uptake and translocation. PtPht1-12 bears the highest sequence similarity to AtPht1-5, a Pi transporter implicated in remobilization, and is upregulated during senescence. PtPht1-5 and 1-6 are the most upregulated during senescence (Loth-Pereda et al., 2011). This favours the targeting of PtPht 1-1, 1-5, 1-6 or 1-12 for downregulation in poplar, with the intent of elucidating the roles of the Pht1 gene family in Pi mobilization during senescence. The mis-regulation would likely have to be done in tandem due to redundant function and overlapping expression patterns of Pi transporters. Any conclusions could tentatively be extended to willow, as Salix spp. are considered to have similar numbers of Pht1 transporters due to the salicoid-specific whole-genome duplication event and the macrosynteny that exists between poplar and willow genomes (Hanley et al., 2006; Puckett et al., 2012). By investigating the function of these transporters and their expression under differing phosphate levels, Canadian poplar and willow varieties could be screened for their ability to limit non-point source Pi entry to freshwater tributaries via biofiltration.

## **1.5** SENESCENCE AND NUTRIENT REABSORPTION IN TREE SPECIES

From a phytoremediation perspective, fast-growing, easily-propagated trees species are ideal for many applications. However, the perennial nature of these crops and the use of leaves as storage organs may reduce the effectiveness of contaminant removal during periods of leaf abscission. From forested ecosystems, windblown leaf litter has been estimated to be 23 % of total phosphorus inputs into tributaries (Wetzel, 2001), making proficient resorption imperative for reducing phosphorus loading from leaves with increased phosphate content.

Autumnal leaf senescence in perennial tree species is a highly regulated process that is initiated by photoperiod and accelerated by low temperatures. It is described as the sequential degradation of cellular enzymes, membranes, and genetic material with the intent of salvaging and mobilizing nutrients predominantly into bark and root tissue to fuel the next year's growth (Fracheboud et al., 2009; Vogel and Dawson, 1993). Leaf senescence has been separated into four distinct phases: bud set and competence to senesce (phase 1), decreasing leaf chlorophyll content (phase 2), no further chlorophyll degradation (phase 3), and the initiation of leaf abscission (phase 4). The majority of leaf weight lost during senescence is attributed to the translocation of carbohydrates with expected declines of up to 35 % of leaf mass area (LMA) (Collier and Thibodeau, 1995) occurring throughout phases 2, 3 and 4 (Keskitalo et al., 2005). When mobilizing nutrients from leaf tissue, nitrogen is primarily translocated in phase 2 as amino acids (82 - 91 %)of recovered nitrogen) and stored as proteins, with a small fraction found as nucleic acids. Phosphorus is mobilized (during phase 1 and 2) as inorganic phosphate and stored primarily as phospholipids, non-hydrolyzable ester phosphate and nucleic acid within buds and stems (Chapin and Kedrowski, 1983; Keskitalo et al., 2005; Kurita et al., 2017). For nitrogen and phosphorus, the two most mobile nutrients in plant tissues, absolute concentrations to which nutrients can be reduced in senescent tissue (resorption proficiency) is 0.3 %N and 0.01 %P. High proficiency is described by reducing absolute concentrations below 0.7 %N and 0.05 %P for deciduous species. Incomplete resorption occurs when leaf concentrations are above 1.0 %N and 0.08 %P (Killingbeck, 1996). Resorption efficiency, describing the relative levels to which perennials can recover nutrients committed to leaves, varies within species, as well as environmental conditions, such as drought and nutrient supply. Poplar trees are able to achieve phosphorus resorption efficiency values ranging between 23 - 60 %, with a positive correlation existing between nitrogen supply and phosphorus resorption efficiency (Brown and Driessche, 2005). Early abscission also

alters resorption efficiency, affecting nitrogen to a greater extent than phosphorus. As trees age, and height and diameter at breast height (DBH) increase, nitrogen resorption decreases significantly while the resorption of phosphorus is unaffected (Killingbeck et al., 1990). This has distinct implications when screening young trees for high phosphorus resorption efficiencies with the intent of extrapolating the data to estimate the performance of multi–year poplar and willow plantations.

For other nutrients found in leaf tissue, resorption is relatively minimal or does not occur. Only ~30 % of leaf potassium is recovered during a short period of time immediately before phase 2 (Keskitalo et al., 2005), and up to 20 % of copper can be mobilized from senescing leaf tissue. Ca, Zn, Fe, Mn, Mg, B and Al are relatively immobile and are discarded as the leaf abscises (Killingbeck et al., 1990). Phytoremediation of Cu, Zn, Fe, Mn and Al would therefore have to take leaf disposal and leaf litter inputs into account, should trees be used on contaminated sites.

The underlying genetic factors and molecular mechanisms of nutrient resorption during senescence are largely unknown. This is in part due to redundancy of genes involved in nutrient mobilization, but also because their role in senescence may be difficult to determine, as these genes are likely essential for normal plant development. In the context of this study, higher order mutants would be required to identify a phenotype during plant development, followed by the insertion of a promoter gene combination to complement the phenotype up until the start of senescence (Himelblau and Amasino, 2001). To my knowledge, this has yet to be done in *Arabidopsis* and such mutants are not feasible in perennial species. For perennials, gene knockdown technologies such as RNA interference (RNAi) or gene knockout systems such as CRISPR/Cas9 would need to be used once optimal candidates were identified. Currently, there are insufficient data with which to select genes that should be targeted as transporters involved in phosphate efflux from cells (Smith et al., 2000). However, the recent discovery of vacuolar phosphate transporters, in particular the low-affinity rice phosphate transporter OsSPX–MFS3 involved in phosphate influx and efflux across the tonoplast (Wang et al., 2015), provides the first step in targeted improvement of phosphate remobilization during senescence.

## **1.6** APPLICATIONS OF SALICACEOUS TREE SPECIES IN CANADA

Trees belonging to the family Salicaceae are well known for their ubiquity throughout Canadian forests and wide-geographical distribution across the continent. This continuous geographic distribution, coupled with high rates of outcrossing and ease with which interspecies hybridization occurs, leads poplar (Populus) and willow (Salix) species to maintain high levels of genetic diversity across their natural range (Isebrands and Richardson, 2014). This diversity has been harnessed since the mid–1900s with the start of hybrid poplar breeding programs in Ontario, Québec and British Columbia aimed at producing varieties more suited for the production of pulp, paper and plywood. A simultaneous breeding program at the Prairie Farm Rehabilitation Administration (PFRA) Shelterbelt Centre in Saskatchewan focused on generating suitable varieties for windbreaks (Thomas and Richardson, 2006; Zufa, 1969). The late 1980s heralded increased interest in developing biomass-accruing willow varieties for bioenergy applications in Eastern Canada and the United States due to Septoria canker limiting hybrid poplar growth, but having little effect on willow biomass production (Kopp et al., 2001). Common traits of Salicaceous tree species, making them amenable to an array of applications, include high-growth rates and productivity, efficient nutrient uptake, ease of propagation and the ability to be coppiced. Current uses of plantation-grown trees range from traditional uses as shelterbelts and fibre production to novel functions as biofilters for phytoremediation and as energy crops (Fortier et al., 2010; Isebrands and Richardson, 2014; Volk et al., 2006).

The biomass produced from energy crops such as hybrid poplar and willow has a variety of uses, either for heating, power generation, or as an alternative feedstock for the production of biofuels, such as ethanol. As an emerging market, lignocellulosic biofuels have the ability to use multi–purpose feedstocks to produce a closed–loop renewable liquid fuel resource grown on marginal land (Murphy et al., 2011; Youngs and Somerville, 2012). Should woody biomass from phosphorus accumulating varieties of hybrid poplar and willow be used for biofuel production, not only would it result in the production of ethanol and/or butanol for liquid fuel, it would also create a biochar containing high levels of phosphorus suitable for use as a fertilizer on phosphorus-deficient fields (Wang et al., 2012). The intended result would be the long–term management of phosphorus levels in both water and soil, while providing landowners with a reliable source of biomass for the production of liquid fuel.
# **1.7 MODEL ORGANISMS**

*Arabidopsis thaliana* L., a small annual species of the mustard family (Brassicaceae), is considered the model organism with which to study complex patterns of development, such as flowering, hormone action and environmental signalling in higher plants. Its broad natural distribution, small stature, short six-week lifecycle and ability to be transformed led to it becoming the first plant to have its genome sequenced (Kaul et al., 2000; Meinke et al., 1998). It is now the most widely–studied plant. The Arabidopsis Information Resource (TAIR) (Rhee et al., 2003) maintains a database of genetic and molecular biology data for the organism as well as access to the Arabidopsis Biological Resource Centre (ABRC) seed collection that contains a wide array of *Agrobacterium* transferred DNA (T–DNA) insertion mutants provided by the Salk Institute (Alonso et al., 2003). These *A. thaliana* insertional mutants are available to researchers around the world and provide a platform with which gene function can be systematically examined. Further transgenic lines can be developed through *Agrobacterium*-mediated transformation using the floral dipping method (Zhang et al., 2006).

Members of the genera *Populus* and *Salix* include a multitude of ecologically and economically important species that collectively have a wide geographical distribution across North America. Their rapid growth, high levels of genetic diversity and ease with which they can be propagated are all attributes which make them ideal model organisms for researching woody perennial species (Brunner et al., 2004). The release of the *P. trichocarpa* Torr. & Gray genome (Tuskan et al., 2006) and recent release of the *S. purpurea* L. genome (Department of Energy Joint Genome Institute, 2014) has furthered their utility in exploring the molecular mechanisms controlling tree development using comparative genomics. For *Populus* spp. in particular, there are many genotypes that are easily transformed and exhibit stable transgene expression (Strauss et al., 2004). *Salix* spp. remain recalcitrant to transformation and method optimizations for species indigenous to North America have yet to be described (Yang et al., 2013).

For the aforementioned model organisms, there are a multitude of online tools that can analyze plant genomic, transcriptomic and proteomic datasets. Those used throughout this thesis were Phytozome (Goodstein et al., 2012), an electronic Fluorescent Pictograph (eFP) browser hosted by the University of Toronto (Winter et al., 2007) and the *Populus* Genome Integrative Explorer (PopGenIE) (Sjödin et al., 2009). The widespread availability of these tools allows for

more informed gene selection and a greater ability to address current knowledge gaps in plant physiology and development.

# **1.8 RESEARCH OBJECTIVES**

The research objectives of my thesis are as follows:

- 1. Select optimal Canadian hybrid poplar and willow varieties for use in phytoremediating phosphorus-rich sites (Chapter 2).
- 2. Identify metabolic differences between low and high phosphate-accumulating poplar, and willow genotypes. This includes a comparison of leaf anion concentrations to assess at which stage of senescence (evaluated based on leaf chlorophyll levels) the lowest resorption proficiencies for phosphate are reached (Chapter 3).
- 3. Test T-DNA knockout lines of the Pht1 phosphate transporter family in *A. thaliana* for growth or metabolite-based phenotypes in mature plants, senescent plants and seeds (Chapter 4).
- Quantify the effects of overexpression of an exogenous Pht1 family member in the annual *A. thaliana* and the perennial hybrid poplar (*P. grandidentata* x *alba*) (Chapter 4).

# **1.9 IMPACT OF RESEARCH**

Plant species often demonstrate high levels of intra– and interspecific variation in nutrient acquisition, making the screening of commercially available genotypes crucial for the use of plants in phytoremediation. Phosphorus uptake and resorption has been studied extensively in many annual and woody perennial plant species in the context of phosphorus deficiency and cycling, or with primary focus on mycorrhizal associations. However, interspecies comparisons of plant performance under excess-phosphorus conditions and the resulting effects on internal anion concentrations are not well understood. Establishing a method with which to assess the interdependence and interactions of anions within the plant would identify any potential trade–off(s) required for the storage of excess phosphate. Differences across genotypes in the extent of phosphorus storage and resorption would provide a basis for a comparative genomic study to determine the genetic controls of these traits in perennial species. Once potential gene candidates are identified, the development of transgenic lines would help to pinpoint current barriers of phosphate remobilization during leaf senescence. Should higher resorption rates be achievable in

the phosphorus-accumulating tree species, phytoremediation of phosphorus-ladened soils could be attained by coppicing existing stands and then turning the material into value–added products. This information could extend further into the selective breeding of perennial crops with a reduced reliance on synthetic fertilizer applications due to improved remobilization of phosphorus during senescence.

# 2. PHOSPHORUS STORAGE AND RESORPTION IN RIPARIAN TREE SPECIES: ENVIRONMENTAL APPLICATIONS OF POPLAR AND WILLOW

# 2.1 SUMMARY

Phosphorus is a contaminant of concern in agricultural systems as increased concentrations in runoff have led to escalating incidents of eutrophication. Improved land management has had little effect on reducing the entry of diffuse terrestrial losses of sediment and dissolved phosphorus into riparian ecosystems. Given that the use of poplar and willow hybrids to prevent erosion in agriculture is widespread in Canada, assessing their ability to act as vegetative filters (biofilters) for nutrient contaminants is vital to determining their long–term potential for phytoremediation. To assess the phosphorus storage capabilities of commercially available poplar and willow hybrids, trees were exposed to control, field and excessive levels of phosphorus in two greenhouse trials. Of the poplar and willow hybrids available in Canada, Tristis and Northwest poplar hybrids had the highest capacity for luxury uptake of phosphorus. However, the majority of phosphorus was stored in leaves and failed to be remobilized during leaf senescence. Hybrid willow with high aboveground biomass allocation to the stem, such as AAFC–5, could be an alternative, as coppicing would remove higher amounts of phosphorus from the site. Although luxury uptake was not observed in AAFC–5, it had the highest resorption proficiency of all trees tested, resulting in the lowest return of phosphorus to the surrounding ecosystem during senescence.

## 2.2 MATERIALS AND METHODS

## **2.2.1 GREENHOUSE TRIALS**

Two greenhouse trials were conducted between July 2013 and January 2015 at the University of British Columbia Horticulture Greenhouse. All cuttings used were taken from field– grown trees at the Agriculture and Agri–Foods Canada (AAFC) Agroforestry Development Centre in Indian Head Saskatchewan. The initial screening performed in 2013 consisted of six willow and four poplar genotypes (Table 2.1). Dormant cuttings were removed from 4 °C, and their diameters measured prior to being dipped in a 0.4 % IBA powder (Stim Root No.2) and planted directly into two-gallon pots. Fifty trees per genotype were planted resulting in roughly 16 trees per treatment per genotype. Trees were grown under 18 hour supplemental lighting which provided at least 600 Wm<sup>2</sup> and extra buds were removed to ensure that there was a single stem per cutting. Treatments consisted of one of the following nutrient ratios: (A) 100N:11P:70K:9Ca, (B) 100N:28P:70K:20Ca, (C) 100N:70P:70K:51Ca (mg L<sup>-1</sup>). This represents a range of 335  $\mu$ M – 2260  $\mu$ M P. On a weekly basis the pH of the nutrient solutions, tree height and diameter were recorded and tables were flooded, exposing the appropriate trees to one of the three different N:P ratios. The control treatment was based on established poplar and willow optimal nutrition ratios (Ericsson, 1981; Hui-Jun and Ingestad, 1984), while the subsequent treatments containing increasing phosphorus concentrations were chosen based on observed ranges of excess soil phosphorus in Manitoba (Manitoba Phosphorus Expert Committee, 2006). Optimal nutrient ratios for N:P employed are slightly higher than the average ratio of 100N:8P observed in the foliage of temperate broadleaf forests (McGroddy et al., 2004).

Phosphate was delivered in the form of  $Ca(H_2PO_4)_2$ , allowing only calcium and phosphate concentrations to vary between treatments. The increasing concentration of calcium was considered to be acceptable, as in all treatments, the N:Ca ratio remains lower than is typical of Hoagland's solution (100N:20P:93K:98Ca) (Hoagland and Arnon, 1950).

Willow		Poplar			
Genotype	Parentage	Genotype	Parentage		
India	S. dasyclados x ?	BalsamBC	P. balsamifera (Fort Nelson)		
AAFC 1	S. discolor (LEV–D5) x S. discolor (MOR–D4)	Northwest	P. balsamifera x P. deltoides		
AAFC 2	S. eriocephala (ANN–E5) x S. eriocephala (ARM–E1)	Okanese	P. 'Walker' x P. petrowskyana		
AAFC 3	S. discolor (LEV–D5) x S. dasyclados (India)	Walker	P. deltoides x P. petrowskyana		
AAFC 4	S. discolor (LEV–D5) x S. dasyclados (India)				
AAFC 5	S. discolor (LEV–D5) x S. dasyclados (India)				

TABLE 2.1: LIST OF GENOTYPES USED IN THE 2013 TRIAL

After 100 days of growth, half of the trees were harvested and fresh biomass of stems, leaves and roots were recorded. The 9<sup>th</sup> leaf of each tree, beginning from the first fully unfurled leaf, as well as bark and xylem scrapings were flash-frozen in liquid nitrogen for later analysis. The remaining trees were moved outside and left to senesce naturally from October – December. Once the top leaves on the majority of trees within a single genotype were 50 % yellow

(determined visually), the 9<sup>th</sup> leaf from each tree within that genotype was frozen in liquid nitrogen and any leaves below were stripped from the tree and dried in paper bags. After trees had their leaves removed, all trees were harvested and fresh biomass for the stem and belowground biomass was recorded. Xylem, bark and roots were air–dried, while leaves were oven dried at 50 °C for three days before dry weights were taken. In cases where a total plant dry weight measurement was not possible for individual samples, water content was calculated for each genotype and applied to fresh biomass as an estimate. Four promising genotypes, Northwest, Okanese, AAFC– 5 and AAFC–3 were used for comparative analysis of tissue before and after autumnal senescence.

In the second screening trial, started in July 2014, willow species were excluded as they did not appreciably accumulate excess phosphorus in aboveground tissue. Accordingly, the number of poplar genotypes was expanded to eight (Table 2.2). Fifty trees per genotype were again planted resulting in 10 trees per treatment per genotype. Higher treatment levels and greater numbers of treatments were established to determine a toxicity threshold. As high concentrations of calcium led to the precipitation of phosphorus, phosphoric acid was used to increase the solution concentrations and was buffered to a pH of 5.5 using potassium bicarbonate. Using this method, the ratio of K:P remained constant around 1.5, while the N:P ratio was progressively lower. Treatments consisted of one of the following nutrient ratios; (A) 100N:13P:70K:7Ca, (B) 100N:70P:113K:12Ca, (C) 100N:100P:152K:12Ca, (D) 100N:130P:198K:12Ca, and (E) 100N:195P:291K:12Ca (mg L<sup>-1</sup>). This represents a range of 420  $\mu$ M – 6300  $\mu$ M P.

Each tree was placed within its own watering tray to minimize leaching and arranged in a randomized-block design. On a weekly basis, each tree was given 500 mL of the appropriate treatment solution from the top of the pot. Any excess solution then collected in the tray below, thereby ensuring that the tree remained exposed to the full concentration of phosphorus in relation to the other nutrients present. The control treatment remained equal to what was used in the initial screening.

Poplar					
Genotype	Parentage				
Assiniboine	Open pollinated Walker seedling				
Hill	P. deltoides x P. petrowskyana				
MIN–13	P. balsamifera (Minnedosa)				
Northwest	P. balsamifera x P. deltoides				
Okanese	P.'Walker' x P. petrowskyana				
P39	P. grandidentata x P. alba				
Tristis	P. tristis x P. balsamifera				
Walker	P. deltoides x P. petrowskyana				

TABLE 2.2: LIST OF GENOTYPES USED IN THE 2014 POPLAR TRIAL.

After 11 weeks of growth, half of the trees were harvested and fresh biomass for stems, leaves and roots were recorded. Three leaves, one from the top, middle and bottom of the tree were randomly chosen, weighed and scanned to calculate leaf mass area. The 9<sup>th</sup> leaf, counting from the first fully unfurled leaf, from each tree, as well as bark and xylem scrapings, were frozen in liquid nitrogen for later analysis.

The remaining trees were wrapped in deer netting and moved outside from mid-Sept to January. Every two weeks, the leaves that had fallen off the trees naturally were collected from the bottom of the netting. Subsequently, 1 - 3 leaves from the batch were scanned for leaf mass area. Leaves were allowed to air dry on tables for 1 week before being placed in labeled paper bags to dry. When the 9<sup>th</sup> leaf on the majority of trees within a genotype was visually assessed to be > 60 % yellow, a chlorophyll concentration index (CCI) measurement was taken from the top right–hand side of the leaf using a Opti–Sciences CCM200plus chlorophyll meter prior to the leaf being flash-frozen in liquid nitrogen. These procedures continued until all trees were devoid of leaves. This methodology allowed for the eventual calculation of resorption efficiency by dividing the difference between total leaf-phosphorus content of mature leaves and senescent leaves by the total content of mature leaves. After trees had dropped their leaves, all trees were harvested and fresh biomass of the stem and belowground biomass was recorded.

#### **2.2.2 CHEMICAL ANALYSES**

#### 2.2.2.1 SAMPLE PREPARATION AND ELEMENTAL ANALYSIS

Using total fresh biomass as the distinguishing parameter, the three individuals closest to the treatment median for each genotype were chosen to have their tissues dried then ground using the 2010 GenoGrinder<sup>®</sup> tissue homogenizer (SPEX<sup>®</sup> SamplePrep) at 1600 rpm for 30 – 60 seconds depending on the tissue. For leaves and roots, the entire organ was pooled and ground while the bottom 10 cm of the tree was collected for xylem and bark. Ground tissue was sent to SGS Laboratories in Guelph Ontario for determination of %N, P, K, Ca, Mg, according to the Association of Official Analytical Chemists (AOAC) protocol 985.01, with concentrations being determined by inductively coupled plasma atomic emission spectroscopy (ICP–AES) (Helrich, 1990).

#### 2.2.2.2 Cell WALL CHARACTERISTICS

Subsamples of the ground xylem were used to obtain values for soluble lignin, insoluble lignin and structural sugars through a modified Klason procedure developed in the Mansfield lab. For the Klason micro-method, 10 mg of dried, acetone-extracted xylem was incubated in 0.1 mL of 72 % sulfuric acid at 30 °C for 1 hour at 500 rpm. 2.0 mL of deionized water was added to each reaction and samples were autoclaved at 140 °C for 75 minutes. Samples were cooled then spun at 20 000 rpm for 5 minutes and the supernatant was transferred to a new microcentrifuge tube. The 1.0 mL of supernatant was then passed through a 0.45 µm filter into a sample vial for structural sugar separation using high-performance liquid chromatography (HPLC). A mixture of peroxide and sulfuric acid at a 1:10 ratio was prepared and 0.5 mL added to the pellet. The reaction was then incubated at 70 °C for 50 minutes, and the sample vortexed after each 25 minute interval. Samples were cooled and spun for one minute to remove bubbles. The UV absorbance of samples was diluted with deionized water until measurements fell within the acceptable range of 0.3 - 0.7AU. Insoluble lignin content was estimated by absorbance at 280 nm and soluble lignin at 205 nm. Structural sugars in HPLC samples were separated using a Dionex CarboPac PA1 column and eluted with deionized water at a flow rate of 1 mL min<sup>-1</sup>. Post–column addition of 200 mM NaOH at a flow rate of 0.5 mL min<sup>-1</sup> and a pulsed amperometric detector with a gold electrode was used for detection.

## 2.2.2.3 PHOSPHATE DETERMINATION

The 9<sup>th</sup> leaf collected from each tree during harvest was used for inorganic phosphate determination using high performance liquid chromatography. Inorganic phosphate was extracted by boiling 10 mg of dried ground tissue in 0.5 mL of 0.5 M HCl for 15 minutes. Samples were then centrifuged for 10 minutes at 17,000 g. The supernatant was placed into a fresh tube and diluted to four times the initial volume with deionized water. 20  $\mu$ L of sample was injected and separated on an IonPac AS–11 (4 x 250 mm) column at 30 °C. The elution profile ran from 0 – 3 minutes isocratic at 5 mM of NaOH followed by 3 – 15 minutes at a linear gradient of NaOH increasing from 5 – 100 mM. At the end of each run, the column was washed with 0.3 – 0.5 M NaOH for 15 minutes followed by a 20 minute equilibration at 5 mM. Flow rates were a constant 1 mL min<sup>-1</sup> with anion concentrations determined using a conductivity detector. Background conductivity was decreased using an anion self–regenerating suppressor (Bentsink et al., 2002; Zhao et al., 2008). Further details on the HPLC methodology can be found in section 3.2.2.

#### **2.2.3 STATISTICAL ANALYSIS**

R version 3.3.2 (R Core Team, 2016) was used to fit linear mixed–effects models with the packages lmerTest (Kuznetsova, 2016) and nlme (Pinheiro et al., 2016). ANOVA was used to compare various models and to test the significance of the categorical predictors and their interactions. Multiple comparisons were done using the Tukey method as a post-hoc test to identify significance between sample means. Bland–Altman analysis for method comparison was done using the package MethComp (Carstensen et al., 2015).

# 2.3 **RESULTS**

## **2.3.1 BIOMASS PRODUCTION AND CELL WALL COMPOSITION**

Lack of an observable effect on growth traits and biomass across increasing levels of applied soluble phosphate confirmed that at all ratios of phosphate application, phosphorus was in excess to what is required for continued growth. None of the genotypes in either trial showed significant changes in total biomass or in proportional biomass allotted to different plant organs across treatments. All hybrids, regardless of genus, generated more biomass than non-hybrid genotypes. Biomass partitioning varied between genotypes, with the majority of AAFC willow hybrids allocating greater amounts of biomass to the stem, while poplar hybrids tended to have higher leaf biomass (Table 2.3).

Increasing soluble phosphate treatments had no effect on lignin or cell wall structural sugar composition in any genotypes employed in the 2013 greenhouse trial. Across genotypes, soluble and total lignin ranged from 2.5 - 3.5 % and 23.4 - 26.3 % respectively. 9 out of 10 genotypes had total lignin concentrations above 25 %, while BalsamBC had a measured total lignin of 23.4 %. Cellulose and hemicellulose values ranged from 47.4 - 51.4 % and 20.5 - 23.3 % dry weight of acetone-extracted xylem tissue across the genotypes. Okanese had the highest percent of cellulose at 51.4 %, while Northwest had the highest percent of hemicellulose at 23.3 %.

2013						2014					
Genotype	Hybrid	Biomass (g)	Stem (%)	Leaves (%)	Roots (%)	Genotype	Hybrid	Biomass (g)	Stem (%)	Leaves (%)	Roots (%)
AAFC-4	Y	330.0 <sup>abc</sup>	41.5 <sup>c</sup>	37.7 <sup>d</sup>	$20.8^{\mathrm{f}}$	Tristis	Y	258.1ª	27.5 <sup>bc</sup>	26.9 <sup>f</sup>	45.7 <sup>a</sup>
AAFC-5	Y	329.1 <sup>abc</sup>	41.6 <sup>c</sup>	38.2°	$20.2^{f}$	P39	Y	256.1ª	22.7 <sup>d</sup>	34.3 <sup>d</sup>	43.1 <sup>ab</sup>
India	_	312.4 <sup>abc</sup>	29.5 <sup>g</sup>	31.6 <sup>f</sup>	38.9 <sup>a</sup>	Okanese	Y	251.2ª	28.8 <sup>b</sup>	30.8 <sup>e</sup>	40.3 <sup>b</sup>
Walker	Y	308.0 <sup>abcd</sup>	37.8 <sup>d</sup>	44.3 <sup>a</sup>	17.8 <sup>g</sup>	Assiniboine	Y	233.0 <sup>ab</sup>	32.0 <sup>a</sup>	34.9 <sup>cd</sup>	33.1°
AAFC-3	Y	285.3 <sup>bcde</sup>	35.9 <sup>e</sup>	41.9 <sup>b</sup>	22.2 <sup>e</sup>	Hill	Y	222.0 <sup>bc</sup>	25.3 <sup>cd</sup>	38.1 <sup>bc</sup>	36.6°
Okanese	Y	272.6 <sup>cde</sup>	29.3 <sup>gh</sup>	34.5 <sup>e</sup>	36.2 <sup>b</sup>	Northwest	Y	210.9 <sup>bc</sup>	28.6 <sup>b</sup>	34.8 <sup>d</sup>	36.5°
Northwest	Y	271.4 <sup>de</sup>	$34.7^{\mathrm{f}}$	38.7°	26.6 <sup>c</sup>	Walker	Y	201.4 <sup>c</sup>	33.6 <sup>a</sup>	40.8 <sup>b</sup>	25.6 <sup>d</sup>
AAFC-2	Ν	$230.4^{f}$	45.5 <sup>b</sup>	34.2 <sup>e</sup>	20.3 <sup>f</sup>	MIN-13	Ν	128.4 <sup>d</sup>	33.2 <sup>a</sup>	46.8 <sup>a</sup>	20.0 <sup>e</sup>
BalsamBC	Ν	157.0 <sup>g</sup>	54.8 <sup>a</sup>	21.5 <sup>g</sup>	23.7 <sup>d</sup>						
AAFC-1	Ν	128.7 <sup>g</sup>	28.1 <sup>h</sup>	32.3 <sup>f</sup>	39.6 <sup>a</sup>						

TABLE 2.3: RANKING OF THE 2013/14 GENOTYPES BASED ON MEAN BIOMASS FROM HIGHEST TO LOWEST. LETTERS REPRESENT SIGNIFICANCE (P < 0.05).

#### 2.3.2 PARTITIONING AND STORAGE OF PHOSPHORUS

Prior to the initiation of autumnal senescence, leaves were the primary storage organ for phosphorus, followed by roots and xylem. Willow hybrids AAFC–3, AAFC–4 and AAFC–5 had similar concentrations in both leaves and roots while all other genotypes had higher phosphorus concentrations in leaves. Both organs demonstrated trends of increasing concentrations as external soluble phosphate increased. Xylem had a low, constant concentration in all genotypes across treatments (Fig. 2.1). When biomass allocation is considered, external phosphate levels had minimal effect on the content of phosphorus content, most notably in Northwest, which had a significantly higher leaf phosphorus content at 100N:70P, compared to the 100N:11P control treatment. Phosphorus content in roots and xylem remained constant across treatments for all genotypes (Fig. 2.2).

Two phosphate-accumulating hybrid poplars (Northwest and Okanese) and two hybrid willows (AAFC–3 and AAFC–5) were further examined for evidence of differential storage across phosphorus treatments and to assess their resorption capabilities. In mature leaves, Okanese was the only genotype to demonstrate a significant shift in the location of phosphorus storage, with an increasing proportion of plant total phosphorus being allocated to leaves under 100N:70P conditions (Table 2.4A). During autumnal senescence in the control treatment, all four genotypes remobilized phosphorus from leaves to roots with marginal changes to the proportion of total phosphorus allocated to the xylem or bark. Under high external phosphate loading, the shift to roots still occurred but in hybrid poplar it had been significantly reduced with most of total plant phosphorus remaining in the leaves (Table 2.4A). Phosphate treatments had the greatest effect on leaf phosphorus concentrations regardless of senescence stage (Table 2.4B). Interestingly, when total plant content of phosphorus is compared before and after autumnal senescence, the willow genotypes had similar content while the poplar hybrids had significantly higher plant phosphorus content after leaf senescence, indicating continued uptake after shoot growth cessation and bud–set.



FIGURE 2.1: MEAN PHOSPHORUS CONCENTRATIONS (± SEM) IN THREE DIFFERENT TREE ORGANS, LEAVES, ROOTS AND XYLEM AS IT DIFFERS BASED ON GENOTYPE. A, B and C are the three phosphorus treatments, 100N:11P, 100N:28P, and 100N:70P respectively. All three tissues from nine individual trees (three per treatment) per genotype were submitted for nutrient analysis.



FIGURE 2.2: MEAN PHOSPHORUS CONTENT (± SEM) OF THREE TISSUES ACROSS AN INCREASING SOLUBLE PHOSPHATE GRADIENT (N = 3). HYBRIDS INCLUDE INDIA, AAFC-3, NORTHWEST, OKANESE AND WALKER. AAFC-4 AND AAFC-5 HAVE BEEN LEFT OUT DUE TO SIMILARITY WITH AAFC-3.

TABLE 2.4: (A) SHOWS THE PERCENT OF TOTAL PHOSPHORUS STORED IN EACH SECTION OF THE TREE. (B) INDICATES THE CONCENTRATIONS IN EACH SECTION OF THE TREE. BOLDED NUMBERS REPRESENT SIGNIFICANT DIFFERENCES (P < 0.05) between the treatments. Asterisks (\*) represent significant differences between senescence stages.

А		P	re-senesce	nce			P	ost-senesce	nce		
			100N:11F	)		100N:11P					
		]	Percent of to	otal phosph	orus		]	Percent of to	otal phospho	orus	
	Genotype	Leaves	Roots	Bark	Xylem	Genotype	Leaves	Roots	Bark	Xylem	
	AAFC-3	57.9	15.1	13.3	13.8	AAFC-3	33.5*	31.6*	17.0	17.8	
	AAFC-5	55.5	16.1	13.4	15.0	AAFC-5	21.0*	40.8*	21.2	17.1	
	Northwest	68.8	14.7	10.1	6.4	Northwest	43.6*	42.2*	7.3	6.9	
	Okanese	53.4	31.6	7.3	7.7	Okanese	32.5*	50.9*	8.1	8.5	
			100N:70F					100N:70P			
		]	Percent of to	otal phosph	orus		]	Percent of to	otal phospho	orus	
	Genotype	Leaves	Roots	Bark	Xylem	Genotype	Leaves	Roots	Bark	Xylem	
	AAFC-3	54.1	17.1	15.8	13.0	AAFC-3	38.6*	27.3*	17.8	16.3	
	AAFC-5	51.2	14.1	13.9	20.8	AAFC-5	21.2*	44.4*	17.5	16.9	
	Northwest	72.9	13.3	8.2	5.6	Northwest	58.9*	24.7*	8.2	8.1	
	Okanese	61.5	24.2	7.3	7.0	Okanese	50.1*	37.0*	5.9	7.0	
	P.						Doct composition				
		n					п				
В		P	re-senesce	nce			P	ost-senesce	nce		
В		P	re-senescer 100N:11F	nce			P	ost-senesce 100N:11P	nce		
В		P	Pre-senescer 100N:11F mg P g <sup>-1</sup>	nce			P	ost-senesce 100N:11P mg P g <sup>-1</sup>	nce		
В	Genotype	P	<b>Pre-senescer</b> 100N:11F mg P g <sup>-1</sup> Roots	nce Bark	Xylem	Genotype	Pe	ost-senesce 100N:11P mg P g <sup>-1</sup> Roots	nce Bark	Xylem	
В	Genotype AAFC-3	P Leaves 0.33	re-senescer 100N:11F mg P g <sup>-1</sup> Roots 0.30	Bark 0.19	Xylem 0.09	Genotype AAFC-3	Pe Leaves 0.18*	ost-senesce 100N:11P mg P g <sup>-1</sup> Roots 0.28	nce Bark 0.22	Xylem 0.08	
В	Genotype AAFC-3 AAFC-5	P Leaves 0.33 0.34	Tre-senescer     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24	Bark 0.19 0.16	Xylem 0.09 0.08	Genotype AAFC-3 AAFC-5	Pe Leaves 0.18* 0.15*	ost-senesce 100N:11P mg P g <sup>-1</sup> Roots 0.28 0.27	nce Bark 0.22 0.18	Xylem 0.08 0.09	
В	Genotype AAFC-3 AAFC-5 Northwest	Leaves 0.33 0.34 0.53	Pre-senescer     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27	Bark 0.19 0.16 0.17	Xylem 0.09 0.08 0.07	Genotype AAFC-3 AAFC-5 Northwest	Pe Leaves 0.18* 0.15* 0.73*	ost-senesce 100N:11P mg P g <sup>-1</sup> Roots 0.28 0.27 0.27	Bark     0.22     0.18     0.16	Xylem 0.08 0.09 0.10*	
В	Genotype AAFC-3 AAFC-5 Northwest Okanese	P Leaves 0.33 0.34 0.53 0.42	Pre-senescent     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     0.27	Bark 0.19 0.16 0.17 0.14	Xylem 0.09 0.08 0.07 0.07	Genotype AAFC-3 AAFC-5 Northwest Okanese	Leaves 0.18* 0.15* 0.73* 0.53	ost-senesce 100N:11P mg P g <sup>-1</sup> Roots 0.28 0.27 0.27 0.27 0.27	Bark     0.22     0.18     0.16     0.21*	Xylem 0.08 0.09 0.10* 0.08	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese	P Leaves 0.33 0.34 0.53 0.42	Pre-senescer     100N:11IF     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     0.27     100N:70F	Bark 0.19 0.16 0.17 0.14	Xylem 0.09 0.08 0.07 0.07	Genotype AAFC-3 AAFC-5 Northwest Okanese	Leaves 0.18* 0.15* 0.73* 0.53	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.27     0.27     0.27	Bark     0.22     0.18     0.16     0.21*	Xylem 0.08 0.09 0.10* 0.08	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese	P Leaves 0.33 0.34 0.53 0.42	Pre-senescer     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     100N:70F     mg P g <sup>-1</sup>	Bark 0.19 0.16 0.17 0.14	Xylem 0.09 0.08 0.07 0.07	Genotype AAFC-3 AAFC-5 Northwest Okanese	Leaves 0.18* 0.15* 0.73* 0.53	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.27     0.27     mg P g <sup>-1</sup>	Bark 0.22 0.18 0.16 0.21*	Xylem 0.08 0.09 0.10* 0.08	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype	P <u>Leaves</u> 0.33 0.34 0.53 0.42 Leaves	Pre-senesce     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     100N:70F     mg P g <sup>-1</sup> Roots	Bark     0.19     0.16     0.17     0.14     Bark	Xylem 0.09 0.08 0.07 0.07 Xylem	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype	Pe Leaves 0.18* 0.15* 0.73* 0.53 Leaves	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.27     0.27     0.27     0.27     0.27     0.27     0.27	Bark     0.22     0.18     0.16     0.21*	Xylem 0.08 0.09 0.10* 0.08 Xylem	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3	P Leaves 0.33 0.34 0.53 0.42 Leaves 0.39	Pre-senesce     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     100N:70F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     0.27     100N:70F     mg P g <sup>-1</sup> Roots     0.39	Bark     0.19     0.16     0.17     0.14     D	Xylem 0.09 0.08 0.07 0.07 Xylem 0.10	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3	Pe Leaves 0.18* 0.15* 0.73* 0.53 Leaves 0.26*	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.37	Bark     0.22     0.18     0.16     0.21*	Xylem 0.08 0.09 0.10* 0.08 Xylem 0.11	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3 AAFC-5	P Leaves 0.33 0.34 0.53 0.42 Leaves 0.39 0.38	Pre-senesce     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     100N:70F     mg P g <sup>-1</sup> Roots     0.30     0.23	Bark     0.19     0.16     0.17     0.14     D	Xylem 0.09 0.08 0.07 0.07 Xylem 0.10 0.11	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3 AAFC-5	Pe Leaves 0.18* 0.15* 0.73* 0.53 Leaves 0.26* 0.15*	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.37     0.29	Bark     0.22     0.18     0.16     0.21*	Xylem 0.08 0.09 0.10* 0.08 Xylem 0.11 0.08	
B	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3 AAFC-5 Northwest	P Leaves 0.33 0.34 0.53 0.42 Leaves 0.39 0.38 0.87	Pre-senescer     100N:11F     mg P g <sup>-1</sup> Roots     0.30     0.24     0.27     100N:70F     mg P g <sup>-1</sup> Roots     0.30     0.23     0.39     0.33     0.36	Bark 0.19 0.16 0.17 0.14 Bark 0.26 0.18 0.17	Xylem 0.09 0.08 0.07 0.07 Xylem 0.10 0.11 0.07	Genotype AAFC-3 AAFC-5 Northwest Okanese Genotype AAFC-3 AAFC-5 Northwest	Leaves 0.18* 0.15* 0.73* 0.53 Leaves 0.26* 0.15* 1.13	ost-senesce     100N:11P     mg P g <sup>-1</sup> Roots     0.28     0.27     0.32	Bark     0.22     0.18     0.16     0.21*     Bark     0.25     0.19     0.20	Xylem 0.08 0.09 0.10* 0.08 Xylem 0.11 0.08 0.12*	

#### **2.3.3 PHOSPHORUS RESORPTION**

In the initial trial, irrespective of the treatment, hybrid willows had a respectable proficiency value while hybrid poplar demonstrated a lack thereof, with higher concentrations of phosphorus in senescent leaves (Fig. 2.3). To verify the lack of resorption in poplar and to determine the limits of leaf luxury consumption, additional hybrid poplar genotypes were screened in 2014. At control levels, all poplar hybrids had similar phosphorus content, but as soluble phosphorus application increased, the genotypes Assiniboine, Hill, Northwest, and Tristis showed the highest uptake capabilities (Fig. 2.4). Of the poplar hybrids in this study, only Walker, Assiniboine, Hill, and Tristis demonstrated a capacity for phosphorus resorption during senescence with 12.0 %, 17.9 %, 30.2 %, and 31.7 % resorption efficiencies, respectively. The remaining genotypes displayed similar phosphorus content in leaves before and after senescence. All poplar genotypes, hybrid or otherwise, had senescent leaf phosphorus concentrations greater than or equal to levels found in actively growing, green leaves (Fig. 2.5).







FIGURE 2.4: THE MEAN PHOSPHORUS CONTENT (± SEM) IN GRAMS FOUND IN POPLAR AND POPLAR HYBRID LEAVES ACROSS INCREASING SOLUBLE PHOSPHATE CONCENTRATIONS. ONE LINE REPRESENTS MATURE LEAVES, WHILE THE OTHER IS SENESCENT LEAF TISSUE.



Figure 2.5: Mean phosphorus concentrations ( $\pm$  SEM) in poplar leaf tissue in mature and senescent leaves (n = 15). Asterisks (\*) indicate p < 0.05.

#### **2.3.4 PHOSPHATE AS THE MAIN FORM OF STORAGE**

Percent leaf phosphorus, calculated based on HPLC values of inorganic phosphate, were compared to total leaf phosphorus (%) as determined by ICP-AES using a Bland-Altman analysis. Under optimal to excess conditions, inorganic phosphate concentrations explain 96.3 % of the variation seen in total phosphate of mature leaf tissue (Fig. 2.6A). As such, inorganic phosphate represents the largest fraction of phosphorus in leaves and can be used to infer total phosphorus amounts by adding 0.113 (Fig. 2.6B). Units were mg P per gram of dried tissue.



FIGURE 2.6: BLAND-ALTMAN PLOT SHOWING THE CORRELATION (A) AND THE DIFFERENCE BETWEEN %P CALCULATED FROM HPLC INORGANIC PHOSPHATE CONCENTRATIONS AND %P DETERMINED BY ICP-AES WITH THE CORRESPONDING 95 % CONFIDENCE INTERVAL (B) WITH AN N = 60.

## 2.3.5 REMAINING MACRONUTRIENTS

Among the four genotypes chosen for further examination (AAFC–3, AAFC–5, Northwest and Okanese), increasing phosphate treatments had no effect on the content or concentration of nitrogen, potassium, calcium or magnesium. It is important to note that across the 100N:11P to 100N:70P treatments, only phosphorus and calcium increased, with the N:Ca ratio remaining well below what is found in Hoagland's solution. In the 2014 poplar hybrid trial, only potassium

concentrations had an increasing trend across treatments. This is to be expected given that potassium was used as the primary counter-ion and was required in large amounts at higher treatments to maintain the pH and solubility of the nutrients in solution. Differences were seen in terms of nutrient storage and resorption across genotypes in both trials.

# 2.3.5.1 NITROGEN

Differences in nitrogen storage in mature tissues between poplar and willow hybrids were minimal. Nitrogen was primarily stored in the leaves with bark, roots and xylem as secondary storage organs. Poplar hybrids tended to have more nitrogen stored in root tissue, while for willow hybrids slightly more was found in the xylem. This was most likely due to differential biomass partitioning. Unlike phosphorus, during leaf senescence nitrogen was moved from the leaves and stored in the roots, bark and xylem. Allocation of nitrogen after leaf senescence was of greater interest as poplar hybrids had a higher resorption potential and moved a greater proportion out of the leaves for storage in the roots (Table 2.5). Resorption proficiencies of poplars were < 1.0%N while willows were > 1.0%N. Among the 2014 poplar genotypes, leaf nitrogen resorption efficiencies were highest for Okanese and Tristis, with 72.1 % and 69.4 % respectively. P39 had the lowest resorption efficiency of 37.8 % (Table 2.6).

# 2.3.5.2 POTASSIUM

Much like nitrogen and phosphorus, potassium was predominantly stored in the leaves; however, a much smaller proportion of total plant content was remobilized during senescence. When remobilized, potassium was moved into the roots and there was very little change in the proportion found in other organs (Table 2.5). Tristis, Northwest and Okanese had the highest potassium resorption efficiencies ranging from 49.9 - 62.1 % while P39 was among the lowest at 41.2 % (Table 2.6).

TABLE 2.5: PERCENT OF TOTAL NITROGEN, POTASSIUM, CALCIUM AND MAGNESIUM STORED IN TREE ORGANS AS DETERMINED BY ICP-AES (n = 8). Bolded numbers REPRESENT SIGNIFICANT DIFFERENCE (P < 0.05) between the senescence stages. Letters (a - d) represent statistical differences between genotypes (P < 0.05). Due to low tissue amounts, AAFC-3 and AAFC-5 pre-senescence nitrogen content and AAFC-5 senescent leaf nitrogen content are estimates.

Nitrogen					Potassium					
Pre-senescence					Pre-senescence					
Percent of total nitrogen					Percent of total potassium					
Genotype	Leaves	Roots	Xylem	Bark	Genotype Leaves Roots Xylem Bark					
AAFC-3	74.5 <sup>a</sup>	6.3 <sup>b</sup>	7.0 <sup>a</sup>	12.3 <sup>a</sup>	AAFC-3 $70.7^{a}$ $6.4^{b}$ $9.2^{a}$ $13.7^{a}$					
AAFC-5	$75.8^{a}$	6.4 <sup>b</sup>	7.7 <sup>a</sup>	10.1 <sup>b</sup>	AAFC–5 71.3 <sup>a</sup> 7.4 <sup>b</sup> 9.3 <sup>a</sup> 11.9 <sup>ab</sup>					
Northwest	74.3 <sup>a</sup>	10.0 <sup>a</sup>	4.6 <sup>b</sup>	11.1 <sup>ab</sup>	Northwest 71.6 <sup>a</sup> 12.0 <sup>a</sup> 3.6 <sup>b</sup> 12.8 <sup>ab</sup>					
Okanese	73.5 <sup>a</sup>	12.2 <sup>a</sup>	5.3 <sup>b</sup>	9.1 <sup>b</sup>	Okanese $70.6^{a}$ $15.5^{a}$ $3.7^{b}$ $10.2^{b}$					

Post-senescence					Post-senescence				
Percent of total nitrogen				Percent of total potassium					
Genotype	Leaves	Roots	Xylem	Bark	Genotype	Leaves	Roots	Xylem	Bark
AAFC-3	<b>44.2</b> <sup>a</sup>	<b>19.4</b> <sup>d</sup>	15.9 <sup>ab</sup>	<b>20.6</b> <sup>b</sup>	AAFC-3	65.4 <sup>a</sup>	15.5 <sup>c</sup>	7.0 <sup>ab</sup>	12.1 <sup>ab</sup>
AAFC-5	<b>29.5</b> <sup>b</sup>	<b>25.9</b> <sup>c</sup>	<b>16.7</b> <sup>a</sup>	<b>27.9</b> <sup>a</sup>	AAFC-5	<b>54.7</b> <sup>a</sup>	<b>22.2</b> <sup>b</sup>	8.6 <sup>a</sup>	14.6 <sup>a</sup>
Northwest	<b>17.5</b> <sup>c</sup>	<b>47.5</b> <sup>b</sup>	13.8 <sup>bc</sup>	<b>21.2</b> <sup>b</sup>	Northwest	59.2 <sup>a</sup>	<b>21.3</b> <sup>b</sup>	<b>5.9</b> <sup>b</sup>	13.7 <sup>a</sup>
Okanese	<b>12.4</b> <sup>d</sup>	<b>58.0</b> <sup>a</sup>	<b>11.7</b> <sup>c</sup>	<b>17.9</b> <sup>b</sup>	Okanese	<b>55.2</b> <sup>a</sup>	<b>31.5</b> <sup>a</sup>	3.4 <sup>c</sup>	9.9 <sup>b</sup>

Calcium						Magnesium				
Pre-senescence						Pre-senescence				
	Percent o	of total cal	cium			Percent of	of total mag	gnesium		
Genotype	Leaves	Roots	Xylem	Bark	Genotype	Leaves	Roots	Xylem	Bark	
AAFC-3	72.0a	5.4d	2.7b	19.8bc	AAFC-3	84.7a	4.3c	3.5b	7.6b	
AAFC-5	58.6a	7.6c	3.8a	30.0a	AAFC-5	76.9a	5.8c	5.9a	11.4a	
Northwest	59.8a	13.2b	3.2ab	23.8ab	Northwest	77.4a	8.0b	1.9c	12.8a	
Okanese	63.7a	18.3a	2.7b	15.2c	Okanese	74.9a	10.9a	1.6c	12.6a	
	Post-	-senescen	ce			Post	-senescen	ice		
	Percent o	of total cal	cium			Percent o	of total mag	gnesium		
Genotype	Leaves	Roots	Xylem	Bark	Genotype	Leaves	Roots	Xylem	Bark	
AAFC-3	77.4a	6.6d	<b>1.9c</b>	14.2bc	AAFC-3	78.3a	7.3c	4.7b	9.8a	
AAFC-5	61.1ab	13.3c	2.5b	23.2a	AAFC-5	63.7ab	15.2b	8.2a	12.9a	
Northwest	56.7bc	20.8b	3.9a	18.6b	Northwest	61.5ab	<b>19.6b</b>	7.0a	11.9a	
Okanese	53.6c	<b>30.7</b> a	2.7b	13.0c	Okanese	51.4b	29.6a	6.8a	12.2a	

	<b>Resorption efficiencies</b>				
Genotype	Nitrogen	Potassium			
Assiniboine	$62.1\pm3.9$	$44.8\pm2.8$			
Hill	$60.0\pm4.5$	$48.8\pm4.8$			
MIN-13	$49.4\pm8.3$	$20.9\pm20.4$			
Northwest	$58.1\pm3.7$	$49.2\pm10.3$			
Okanese	$72.1\pm4.2$	$59.9 \pm 12.0$			
P39	$37.8\pm7.5$	$41.3\pm10.6$			
Tristis	$69.4\pm2.7$	$62.1\pm4.6$			
Walker	$58.5\pm8.5$	$44.4\pm9.8$			

TABLE 2.6: LEAF RESORPTION EFFICIENCIES OF POPLAR GENOTYPES AND THEIR ASSOCIATED MARGIN OF ERROR FOR A 95 % CONFIDENCE INTERVAL.

#### 2.3.5.3 CALCIUM AND MAGNESIUM

Both calcium and magnesium were stored in the leaves and the bark with no resorption from the leaves, but there was a slight accumulation in the roots after leaf senescence (Table 2.5). Leaf content of calcium and magnesium did not change across samples taken before and after leaf senescence in any of the poplar genotypes.

#### **2.4 DISCUSSION**

The environmental applications of poplar and willow are limited only by their phenotypic diversity across their natural range. Commonly celebrated traits include rapid growth, high-transpiration rates, and ease of propagation, resulting in the establishment of breeding programs for production of pulp, paper, plywood, and agricultural windbreaks (Thomas and Richardson, 2006; Zufa, 1969). Over the past 20 years, global applications of these trees have shifted towards assessing their value in terms of ecosystem services, such as stream-bank stabilization and excess-nutrient removal (Isebrands and Richardson, 2014). Adding to the knowledge base for such applications becomes imperative for creating sustainable agroecosystems.

#### 2.4.1 BIOMASS, CELL WALL TRAITS AND MACRONUTRIENTS

Lack of significant change in biomass across treatments confirms that the N:P ratios used in this study resulted in conditions where phosphorus was neither toxic nor limiting. It should be noted that the phosphorus treatments applied represent the limits of phosphorus solubility in this artificial system using Ca<sup>2+</sup> and K<sup>+</sup> as counter-ions and holding all other nutrients constant. This held true for hybrid poplar up to applications of 6.3 mM of soluble phosphorus. Micronutrient deficiencies caused by excess phosphorus, such as those previously reported in field studies, were not observed in any treatment (Teng and Timmer, 1990). Unlike the increased cellulose and decreased lignin phenotypes of hybrid poplar treated with high amounts of nitrogen fertilizer (Pitre et al., 2007), hybrid poplar exposed to high soluble phosphate levels did not cause any changes in cellulose or lignin content. None of the other macronutrients tested (N, K, Ca and Mg) showed significant variation in concentration or content caused by the increasing phosphate treatments. Among the hybrids, AAFC–4, AAFC–5, and Walker had the most aboveground biomass (Table 2.3), while both willow and poplar hybrids mobilized phosphorus into the roots for storage upon leaf senescence (Table 2.4). Such traits are important to consider for phytoremediation purposes, as fast–growing tree species can have similar contaminant extraction potential as hyperaccumulators due to their high-biomass production and because coppicing, which generally occurs after leaf senescence, would be the primary method of nutrient removal from the site (Laureysens et al., 2004; Pulford and Watson, 2003).

#### **2.4.2 PHOSPHORUS STORAGE AND ACCUMULATION**

Of the organs tested in Salicaceae species, leaves were affected to the largest extent by external phosphate concentrations and are the best indicators of plant phosphate accumulation. This is consistent with the use of leaves in measuring plant demand for phosphorus in crop plants (Bollons and Barraclough, 1999). Phosphate concentrations in mature willow leaves were similar to values reported in crop tissues grown under optimal phosphate conditions (0.3 - 0.5% P; Marschner 2012), while promising poplar genotypes had leaf concentrations ranging from 0.54 - 0.66 % P (Fig. 2.3).

Leaf phosphorus accumulation has been observed in many different flowering plants, including poplar and willow. Under high phosphorus treatments, grains such as wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) have reported leaf concentrations of 0.1 and 0.3 % P respectively, while field legumes such as peas (*Pisum sativum* L.) and faba beans (*Vicia faba* L.) range from 0.2 - 0.3 % P (Jia et al., 2011; Nuruzzaman et al., 2005). Soybean [*Glycine max* (L.) Merr.] and tobacco (*Nicotiana tabacum* L.) accumulate up to 0.7 % P, while some species native to Australia, such as *Acacia, Banksia* and *Ptilotus* spp. can accumulate anywhere from 1.2 - 4.0

% P (de Campos et al., 2013; Marschner, 2012; Ryan et al., 2009). Above 1.0 % P, the probability of a plant suffering from phosphorus toxicity increases, but visible symptoms may occur below this point in sensitive plants (Bell et al. 1990; Marschner, 2012). At an agriculturally relevant treatment of 100N:70P and at the highest treatment of 100N:195P, the highest accumulating poplar hybrids have leaf concentrations of 0.88 - 1.01 % and 1.5 - 1.8 % P respectively, without toxicity symptoms. Tolerance to high internal phosphate concentrations and vigorous aboveground biomass production is responsible for the higher phosphorus storage potential of these perennial species.

This study further demonstrates that there is a high degree of intraspecies and interspecies variation, where genetic controls allow for luxury consumption in some poplar hybrids, but not in others. This is most notably shown by how the commonality of a *P. balsamifera* parent for Tristis and Northwest does not explain the high phosphorus accumulation in these two genotypes, as neither of the non-hybrid genotypes tested showed accumulation. It is likewise shown by the significantly higher phosphorus accumulation and resorption efficiencies of Assiniboine and Hill, when compared to the related Walker and Okanese genotypes (Fig. 2.4). The overall lack of luxury consumption in the willows (Fig. 2.2) is also of interest as it could provide insight into plant mechanisms for moderating nutrient uptake, when compared to hybrid poplar. This inherent difference could help elucidate the biological mechanism(s) of translocation and phloem loading of phosphate, and consequently identify candidate genes whose expression could later be altered in poplar using biotechnological means.

#### **2.4.3 PHOSPHORUS RESORPTION EFFICIENCY AND PROFICIENCY**

Of the data obtained, there is little evidence to suggest a link between external nutrient availability and resorption in Salicaceous species when phosphorus levels are optimal and above. As treatments increased, the total grams of phosphorus that were resorbed remained relatively constant (Fig. 2.4). In accordance with the findings of Kobe et al. (2005), poplar demonstrated a strong positive linear relationship between mature and senescent leaf phosphorus concentrations across treatments (data not shown). However, mature leaf accumulation levels were not different enough across treatments to significantly affect resorption efficiency. Given the range of N:P ratios used in these experiments, this would support the conclusion that there is a lack of nutritional controls on resorption efficiency (Aerts, 1996). Resorption efficiencies of these experiments

ranged from 35 - 52 % for the willow hybrids and 0 - 32 % for the hybrid poplar, compared to the near 50 % reported in the literature for deciduous forest ecosystems (Aerts, 1996; Vergutz et al., 2012) and 23 - 60 % reported for poplar hybrid plantations (Brown and Driessche, 2005). This puts into perspective not only how variable the process of leaf senescence can be, but also that species and varieties being considered for wide–scale remediation application should be assessed on an individual basis.

Resorption proficiency, the lowest concentration of a nutrient in senesced leaves, is a better tool for cross-species comparisons as it reflects the biochemical limits of nutrient resorption (Killingbeck, 1996). While willow hybrids AAFC–3 and AAFC–5 achieved values of 0.14 - 0.20% phosphorus (Fig. 2.3), poplar genotypes had higher phosphorus concentrations in senescent leaves, ranging between 0.43 – 1.47 % (Fig. 2.3; Fig. 2.5). This indicates incomplete resorption given that senescent leaf phosphorus concentrations were equal or greater than green leaf concentrations. Provided that in plants phosphorus is stored as inorganic phosphate within the cell (Marschner, 2012; Veneklaas et al., 2012; Yang et al., 2017; Fig. 2.6), it stands to reason that the hydrolysis of phosphate esters is not limiting resorption. Sink strength may play a role (Aerts, 1996); however, poplar and willow hybrids had similar phosphorus concentrations in their roots with willow having less root biomass prior to senescence (data not shown). The determining factor may then be the translocation process (Chapin and Moilanen, 1991), where the release of phosphate from the vacuole or phosphate translocation to the phloem is being inhibited in some genotypes. Control mechanisms for phosphate translocation could be determined through gene expression analysis, via RNA sequencing, of hybrid poplar versus hybrid willow. Successfully exploiting variation in phosphorus resorption proficiency among Salicaceae species in breeding programs would have practical applications in the advancement of nutrient conservation in perennial cropping systems and in further minimizing the environmental phosphorus footprint of agricultural production systems.

## **2.4.4 CONCLUSIONS**

In conclusion, the tree species capable of continued luxury uptake of phosphorus had leaf concentrations  $> 5 \text{ mg P g}^{-1}$  as opposed to the 3 – 4 mg P g<sup>-1</sup> observed in low or non-accumulating species at optimal levels of external phosphorus (420  $\mu$ M P). Of the genotypes tested, the poplar hybrid Tristis appears to be the most promising candidate for preventing excess-phosphorus runoff,

due to its larger root systems, higher resorption efficiency among the hybrid poplar and highest luxury consumption of phosphate. Large root systems are ideal, as they provide increased bank stabilization for reduced particulate input into riparian ecosystems and an increased area for interception of dissolved phosphate (Jarvie et al., 2013). Unfortunately, a large percentage of accumulated phosphorus is expected to be re-introduced into the environment upon leaf senescence. Consequently, the use of hybrid willow genotype AAFC-5 would be a good alternative, given that it has higher biomass allocation to the stem and therefore a higher phosphorus content stored within woody tissue than poplar. In the agriculturally relevant treatments 100N:13P and 100N:70P, AAFC-5 had 76 % and 52 % of the phosphate accumulation potential in leaves compared to Tristis but would return 59 % and 70 % less phosphorus to the environment respectively, during leaf senescence. Further investigation into nutrient resorption mechanisms and the underlying differences in leaf nutrient proficiency between poplar and willow is warranted, given that reducing loss of accumulated contaminants through leaf litter is required for efficient use of perennial species in agro-ecological and phytoremediation applications.

## 2.4.5 CONTINUING RESEARCH

Given that the intracellular anion-cation balance must be maintained during excessive anion uptake, both the metabolic differences between high and low phosphorus-accumulating hybrid poplar and the metabolic adjustments that must be made in order to store excess phosphate are of interest.

Furthermore, concentrations and content of phosphorus in senescent leaves were found to be equal to or greater than levels found in mature leaves which may be exacerbated by continued uptake and storage after bud set and should be confirmed. Confirmation of senescence stage at which the greatest resorption proficiency is achieved for varying inorganic anions, organic anions and carbohydrates is of interest, as it may provide insight into existing barriers to resorption of nutrients from tree leaves.

# **3. DISCERNING THE EFFECTS OF PHOSPHATE STATUS ON THE METABOLISM OF HYBRID POPLAR**

# 3.1 SUMMARY

Metabolic responses are often observed when plants are exposed to environmental stressors. For phosphate accumulation in particular, the buffering capacity of the vacuole is required to prevent phosphate from inhibiting starch formation and CO<sub>2</sub> fixation (Marschner, 2012). The excess storage of anions, such as phosphate, must also be charge balanced, generally by using potassium as a counter-ion, as it is not metabolized, or by a reduction in organic acid content (Hiatt, 1967). From the 2014 greenhouse trial, the roots and leaves of four poplar genotypes were tested for differences in initial organic acid and non-structural carbohydrate concentrations, to determine the metabolic effects of increasing external phosphate both before and during leaf senescence. All measured metabolites were found to be largely unaffected by the increasing phosphate concentrations. Leaves from hybrid poplar and willow were further analyzed for differences in inorganic-anion resorption proficiencies and efficiencies. Hybrid poplar demonstrated higher sulfate resorption and hybrid willow higher phosphate resorption, suggesting differing anion remobilization mechanisms. Phosphate uptake was shown to continue well after bud-set in both poplar genotypes, which may contribute to the low phosphorus resorption efficiency values reported in Chapter 2. Observed differences in hybrid poplar resorption efficiencies between Tristis and P39 genotypes led to the comparative analysis of phosphate transporter genes expressed in mature leaves. This allowed for the identification of PHT1-9 and PHT 1-12 as genes potentially involved in the luxury uptake and remobilization of phosphorus.

# **3.2** INTRODUCTION

Facilitated diffusion across plant plasma membranes has been reported for 10 out of 14 mineral nutrients, including many of the cations such as K<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup>, and for organic acids. Inorganic anions such as nitrate, phosphate and sulfate are transported across the plasma membrane against their electrochemical gradient and require active transport, mainly through proton–coupled transporters. At the tonoplast, a similar array of transporters exists, but active transport into the vacuole is required for inorganic cations and nitrate, while movement of phosphate, sulfate and organic acids is through ion channels (Marschner, 2012). It is by controlling

these transport routes that uptake, storage, and the cellular electrochemical equilibrium is maintained.

Organic acid synthesis and decarboxylation are additional methods used by plant cells to adjust and maintain internal pH and electrochemical balances. The catabolism and anabolism of organic acids are suggested to provide pH regulation through the release of  $CO_2$  and the activity of carbonic anhydrase, resulting in buffering by way of bicarbonate (Britto and Kronzucker, 2005; Chang and Roberts, 1992). When excess cations flow into a cell, there is a corresponding increase in cytosolic pH, which is adjusted via the synthesis and carboxylation of organic anions that can be later transported into the vacuole in tandem with the excess cations. In the case of anion influx and decreasing pH, protons are consumed during the decarboxylation of stored organic acids (Britto and Kronzucker, 2005; Hiatt, 1967). While no correlations between K<sup>+</sup> and Pi concentrations in sieve tube members have been shown (Peuke, 2010), increases in leaf K<sup>+</sup> and/or decreases in organic acids concentrations in high-phosphate accumulators is expected.

Baseline data on phosphate resorption proficiencies of suitable genotypes is a required for future assessment of performance in the field. Autumnal leaf senescence in tree species is a highly regulated process of sequential cellular degradation thought to salvage and store nutrients to fuel growth the following year. Leaf senescence progresses through four phases that are initiated by a reduction in photoperiod and accelerated by low temperatures: (1) bud set and competence to senesce, (2) reduction in chlorophyll content, (3) cessation of chlorophyll degradation, and (4) leaf abscission. Phosphorus remobilization (as phosphate) occurs during the first two phases, while soluble sugar depletion occurs from phase two onward (Keskitalo et al., 2005). Among the various poplar species studied in the United States, phosphorus concentrations left in senesced leaves ranged from 0.02 - 0.10 % (Killingbeck, 1996). Compiled data for deciduous species resulted in guidelines dictating that concentrations < 0.05 % P indicated complete resorption, while > 0.08 % P is incomplete (Killingbeck, 1996). As all hybrid poplar used in the previous trials demonstrated senescent leaf concentrations well above these thresholds, the possibility of continued influx of phosphorus into leaves after bud set was explored. For the genotypes best suited for phytoremediation, Tristis and AAFC-5, the resorption proficiencies of inorganic and organic anions throughout the stage of chlorophyll degradation was established.

# **3.3** MATERIALS AND METHODS

#### **3.3.1 GREENHOUSE TRIALS**

#### 3.3.1.1 TRIAL FOR ASSESSMENT OF METABOLIC TRADE-OFFS

The material used for this study was derived from the second greenhouse trial, where the plants were grown in July 2014. Seven hybrid poplar genotypes and one *Populus balsamifera* genotype (Table 2.2) were grown in a randomized block design and treated with five different levels of soluble phosphate. The nutrient ratios for each treatment were as follows; (A) 100N:13P:70K:7Ca, (B) 100N:70P:113K:12Ca, (C) 100N:100P:152K:12Ca, (D) 100N:130P:198K:12Ca, and (E) 100N:195P:291K:12Ca (mg L<sup>-1</sup>). This is a range equivalent to  $420 \,\mu\text{M} - 6300 \,\mu\text{M}$  P. The solutions ranged from pH 5.5 – 6.3.

Trees were given 500 mL of the corresponding nutrient solution every week for 10 weeks, and each tree was placed in its own watering tray to prevent leaching of the phosphate treatment. One week after the final treatment, half of the trees were harvested with the 9<sup>th</sup> leaf from the first fully unfurled leaf (Plastochron Index 9) and a 3 g portion of fine roots were collected and flash-frozen in liquid nitrogen.

The remaining trees were wrapped in deer netting and moved outside to induce natural leaf senescence. When the 9<sup>th</sup> leaf on the majority of trees within a genotype was visually assessed to be > 60 % yellow, a CCI measurement was taken from the top right–hand side of the leaf before storing the sample in liquid nitrogen. Given the variable nature of senescence, the 9<sup>th</sup> leaf could not always be collected. If the 9<sup>th</sup> leaf was unavailable or unsuitable, the closest mature leaf was collected. Leaf numbers were recorded at collection and varied between leaf 5 – 12. Leaves from all trees from a single genotype were collected the same day; however, genotypes were collected on different days starting from October 21<sup>st</sup> to December 2<sup>nd</sup>, 2014. No root tissue was collected into liquid nitrogen after leaf senescence.

## 3.3.1.2 TRIAL FOR ASSESSMENT OF RESORPTION PROFICIENCIES

In conjunction with the second screening, 10 individuals from two poplar (Tristis and P39) and one willow genotype (AAFC–5) were subject to one of two treatments: (A) 100N:13P:70K:7Ca or (B) 100N:70P:113K:12Ca. After 11 weeks, the CCI value of the 9<sup>th</sup> leaf was taken as a pre–senescence baseline, and 80, 60 and 40 % values of that baseline were then calculated. Trees were placed outside to senesce for six weeks. Once the trees had visibly yellow leaves, leaves along the length of the tree were screened based on their chlorophyll estimates. Should a leaf have a value close to 100, 80, 60 or 40 % of the baseline, chlorophyll content was estimated using an Opti–Sciences CCM200plus chlorophyll meter and the associated Plastochron Index was recorded prior to the leaf being frozen in liquid nitrogen. This harvest methodology was repeated for all genotypes.

## **3.3.2** CHEMICAL ANALYSES

#### 3.3.2.1 SAMPLE PREPARATION

For across-treatment comparisons both before and after leaf senescence, four individuals were randomly chosen for tissues analysis. For comparisons along the gradient of chlorophyll degradation, four leaves from five individuals were analyzed. Tissue was kept frozen using liquid nitrogen and ground using the 2010 GenoGrinder® tissue homogenizer (SPEX<sup>®</sup> SamplePrep) at 1600 rpm for 30 seconds for leaves, and  $2 \times 30$  seconds for roots with sample cooling in liquid nitrogen between cycles. Sub–samples of the ground tissue were stored at -80 °C for RNA extractions, and the remainder lyophilized for 20 - 24 hours for organic acid, soluble carbohydrate, and starch determinations.

#### 3.3.2.2 Organic acid and inorganic anion determination

For each biological replicate, two technical replicates of 10 mg were extracted by boiling the lyophilized tissue in 0.5 mL of 0.5 M HCl for 15 minutes. Samples were centrifuged for 10 minutes at 17000 g. 350  $\mu$ L of supernatant was placed into a fresh micro-centrifuge tube and diluted to 1.0 mL with deionized water. The 1.0 mL sample was then passed through a 0.45  $\mu$ m filter into a sample vial for organic acid separation using HPLC (Bentsink et al., 2002; Zhao et al., 2008). HPLC conditions can be found in Fig 3.1, and examples of the resulting chromatograms in Fig 3.2.

Column:	AS11 (4 x 250mm)				
Guard column:	AG11 (4 x 50mm)				
Flow rate:	1 mL min <sup>-1</sup>				
Temperature:	30 °C				
Injection volume:	20 μL				
Detection:	Suppressed conductivity, AERS500 (4mm), AutoSuppression® recycle mode, 250 mA Water 432 Conductivity Detector				
Eluent:	A: 1M NaOH B: Deionized H <sub>2</sub> O				
Gradient:	5 – 500 mM NaOH: 5 mM NaOH, hold for 3 min; 5 – 100 mM in 12 min.				
Method:	Time (min) A (%) B (%)   0 0.5 99.5   3 0.5 99.5   15 10.0 90.0   15.1 30.0 70.0   40 50.0 50.0   40.1 0.5 99.5   60 0.5 99.5				
Typical background conductivity: Typical system operating backpressure:	5 mM NaOH: -0.01 μS 100 mM NaOH: 1.0 - 1.3 μS 1200 psi				

Standard mixed stock		Working standards		
Compound	Weight (g)	Standard	Weight of mixed stock (g)	
D–L– Malic acid	0.092	Н	0.300	
Disodium sulfate anhydrous	0.180	М	0.200	
Oxalic acid dihydrate	0.135	L	0.100	
Sodium phosphate monobasic	0.200	VL	0.050	
Trisodium citrate dihydrate	0.155	VVL	0.025	
Phytic acid dipotassium salt	0.017	VVVL	0.012	
Final volume (g)	50.00	Final volume (g)	1.75	

FIGURE 3.1: OPTIMIZED HPLC PROTOCOL FOR THE DETERMINATION OF ORGANIC ACIDS AND INORGANIC ANIONS USING A DIONEX<sup>TM</sup> FIT WITH AN IONPAC<sup>TM</sup> AS11 COLUMN AND A CONDUCTIVITY DETECTOR.



FIGURE 3.2: CHROMATOGRAMS OF ORGANIC ACID AND INORGANIC ANION SEPARATION ON AN IONPAC<sup>™</sup> AS11 COLUMN. RETENTION TIME SHIFTED SLIGHTLY DUE TO NUMBER OF CONSECUTIVE SAMPLES RUN ACROSS EXPERIMENTS AND CHANGES IN BUFFER SOLUTIONS.

## 3.3.2.3 Soluble Carbohydrate determination

Soluble carbohydrate extractions were completed using 50 mg of lyophilized leaf and root tissue. The dried tissue was weighed into a 15 mL polypropylene tube supplemented with 50  $\mu$ L of a 10 mg mL<sup>-1</sup> galactitol stock added as an internal standard. 4 mL of 12:5:3 methanol, chloroform and water was added, vortexed and left to stand overnight at 4 °C. The following day, samples were centrifuged at 6,000 rpm for 10 minutes and the supernatant transferred into a fresh 50-mL tube. The remaining pellet was resuspended in 4 mL of methanol:chloroform:water, vortexed briefly, and then spun at 6,000 rpm for 10 minutes. The supernatant was again collected into the 50-mL tube. This was repeated three times and the final pooled volume of supernatant stored in the single 50-mL tube. After the final rinse, the pellet was left to dry in a 55 °C oven overnight and used for subsequent starch analysis. The pooled supernatant was phase separated by the addition of 5 mL of deionized water and centrifugation at 4,000 rpm for 4 minutes. The top polar phase was collected into a 15 mL Falcon tube and stored at 4 °C. To prepare the sample for analysis, 2 mL of the polar phase was placed into a micro-centrifuge tube and evaporated to dryness in a vacuum centrifuge (16 - 18 hours). The pellet was then resuspended in 1 mL of deionized water (with the exact weigh of water recorded) and passed through a 0.45-µm filter into an HPLC vial for analysis. Run information can be found in Fig. 3.3 and an example of the resulting chromatograms found in Fig. 3.4. A methods comparison of non-structural carbohydrate extraction protocols, including the one used in this study, can be found in Quentin et al. (2015).

## 3.3.2.4 STARCH DETERMINATION

The dried pellet resulting from the soluble carbohydrate extraction was used in the following procedure. 20 mg of the dried pellet was weighed into a 10-mL glass culture tube and 5 mL of 4 % H<sub>2</sub>SO<sub>4</sub> was added before vortexing. Once all samples were prepared, they were autoclaved for 5 minutes. After cooling, samples were centrifuged for 5 minutes at 500 rpm to pellet the insoluble material. The resulting supernatant was stored in a 15-mL Falcon tube at 4 °C. Samples were prepared for the HPLC analysis by weighing 950  $\mu$ L of supernatant and 50  $\mu$ L of a 15 mg mL<sup>-1</sup> fucose solution to act as the internal standard. Samples were then vortexed briefly and passed through a 0.45- $\mu$ m filter into an HPLC vial. Run information and examples of sample chromatograms can be found in Fig 3.5 and Fig 3.6, respectively.

Column:	PA1 (4 x 250mm)			
Guard column:	PA1 (4 x 50mm)			
Flow rate:	0.8 mL min <sup>-1</sup>			
Temperature:	30 °C			
Injection volume:	15 μL			
Detection:	Post-column, 200 mM NaOH 0.5 mL min <sup>-1</sup> Pulsed amperometric dectection (PAD) gold			
Eluent:	A: 0.2 M NaOH (must be made in a volumetric flask) B: Deionized H <sub>2</sub> O C: N/A			
Gradient:	D: 20 mM NaOAc Isocratic, 16 mM NaOH 2 mM NaOAc			
Method:	Time (min)A (%)B (%)D (%)08.082.010.0308.082.010.030.1100.00.00.040100.00.00.040.18.082.010.0608.082.010.0			
Typical background conductivity:	0.1 – 1.0 nC			
Typical system operating backpressure:	1500 psi			

Standard single stocks		VH Stock		
Compound	Weight (mg)	Final weight (g)	Single stock	Weight (g)
<i>myo</i> -inositol	80	5.0	<i>myo</i> inositol	0.100
Galactose	75	5.0	Galactose	0.050
Glucose	75	5.0	Glucose	0.150
Sucrose	75	5.0	Sucrose	0.300
Fructose	75	5.0	Fructose	0.100
Raffinose pentahydrate	75	5.0	Raffinose pentahydrate	0.100
Stachyose hydrate	75	5.0	Stachyose hydrate	0.100
Galactitol	50	5.0	Final volume (g)	10.0

FIGURE 3.3: Optimized HPLC protocol for the determination of carbohydrates using a Dionex<sup>TM</sup> fit with a CarboPac<sup>TM</sup> PA1 column and an electrochemical detector.



FIGURE 3.4: CHROMATOGRAMS OF SOLUBLE CARBOHYDRATE SEPARATION ON THE CARBOPAC<sup>TM</sup> PA1 COLUMN.
Column:	PA1 (4 x 250mm)				
Guard column:	PA1 (4 x 50mm)				
Flow rate:	1.0 mL min <sup>-1</sup>				
Temperature:	30 °C				
Injection volume:	10 μL				
Detection:	Postcolumn, 200 mM NaOH 0.5 mL min <sup>-1</sup> Pulsed amperometric dectection (PAD) gold				
Eluent:	A: 0.2 M NaOH B: Deionized H <sub>2</sub> O				
Gradient:	N/A				
Method:	Time (min) A (%) B (%)   0 0.0 100.0   35 0.0 100.0   35.1 100.0 0.0   45 100.0 0.0   45.1 0.0 100.0   60 0.0 100.0				
Typical background conductivity:	0.1 – 1.0 nC				
Typical system operating backpressure:	1500 psi				

Standard single stocks			Working standards			
Compound	Weight (mg)	Final (g)	Standard	Glucose stock(g)	Fucose stock (g)	Final (g)
Fucose	75	5.0	VH	0.200	0.020	2.0
Glucose	75	5.0	Н	0.150	0.020	2.0
			М	0.050	0.020	2.0
			L	0.030	0.020	2.0
			VL	0.005	0.020	2.0
			VVL	0.002	0.020	2.0

FIGURE 3.5: DETERMINATION OF GLUCOSE POST ACID HYDROLYSIS OF STARCH USING A CARBOPAC<sup>TM</sup> PA1 COLUMN AND ELECTROCHEMICAL DETECTION.



FIGURE 3.6: CHROMATOGRAMS SHOWING FUCOSE AND GLUCOSE SEPARATION ON THE CARBOPAC<sup>TM</sup> PA1 COLUMN.

## **3.3.3 SEMI-QUANTITATIVE PCR**

Semi-quantitative polymerase chain reaction (SQ-PCR) was chosen over other methods of expression analysis due to the range of genes, tissues and genotypes across which differences were to be assessed. For RNA extraction, PureLink® Plant RNA Reagent (Ambion® by Life Technologies Cat. No. 12322–012) was used. For each extraction, 0.5 mL of cold PureLink<sup>®</sup> Plant RNA Reagent was added to 80 – 90 mg of frozen tissue and vortexed until all tissue was suspended. Tubes were incubated at room temperature for 5 minutes before centrifuging at 12000 g for 2 minutes and transferring the supernatant to a clean RNase-free tube. 0.1 mL of 5 M NaCl was mixed thoroughly into the supernatant followed by an addition of 0.3 mL of chloroform. Samples were then centrifuged at 12,000 g for 10 minutes at 4 °C to facilitate phase separation. The top aqueous phase was transferred to a clean RNase-free tube where an equal volume of isopropyl alcohol was added, and the sample was left at room temperature for 10 minutes. After a final centrifugation at 12,000 g for 10 minutes at 4 °C, the supernatant was decanted and the pellet washed with 1 mL of 75 % ethanol. Samples with concentrations above 200 ng  $\mu L^{-1},$  as determined by an A260/280 absorbance ratio between 1.85 – 2.10 AU, were cleaned using TURBO<sup>™</sup> DNA– free<sup>™</sup> Kit (Ambion<sup>®</sup> by Life Technologies Cat. No. AM 1907) to remove any contaminating DNA. Cleaned RNA was then used to generate cDNA using the iScript<sup>™</sup> Select cDNA Synthesis Kit (BIO-RAD Cat. No. 170-8897). Briefly, 1 µg of RNA template was used for each reaction with  $2 \mu L$  of both Oligo(dT)<sub>20</sub> and random primers for improved cDNA yield, and a greater coverage of potentially degraded transcripts in the resulting cDNA. Reactions were incubated for 5 minutes at 25 °C and then for 60 minutes at 42 °C before heat-inactivation of the reverse transcriptase (5 minutes at 85 °C).

 $1 \ \mu$ L of cDNA was then used for each PCR reaction to estimate gene expression levels of the Pht1 family of genes in mature and senescent leaf tissue of hybrid poplar genotypes Tristis and P39. Due to tissue degradation, root samples did not yield sufficient quality RNA to perform these analyses. Reactions were run with an annealing temperature of 56 °C and an extension time of 25 seconds. Due to low transcript abundance of the transporters, quantification occurred at cycle 35 for Pht1 family genes, while ubiquitin was quantified at cycle 25. Primers employed for the poplar Pht1 were taken from Loth-Pereda et al. (2011) and can be found in Table 3.1.

60

Name	Sequence
PtPht1–1f	gcgattcacgaggtttttca
PtPht1–1r	ggcgaagaaaaaaggtcaacg
PtPht1–2f	cacagaccgaacgaagactg
PtPht1–2r	atcacactgaagccatcctagc
PtPht1-3f	accaccgaattggcttcg
PtPht1-3r	atggcaagtagacctcaatctcg
PtPht1-4f	gatcttccctgcaaggttaagg
PtPht1–4r	tccttgtgcgggctctg
PtPht1-5f	ggacccaatgccaccac
PtPht1–5r	cttggcctggtcctgattc
PtPht1–6f	atcgccagggctcaattc
PtPht1–6r	ctattattgcgccagccttc
PtPht1-7f	cgaattggattcgtggttatg
PtPht1–7r	ccttgtcctggtcctgagc
PtPht1–9f	tggtgactacccctctctg
PtPht1–9r	aggcaccgatccaaacatc
PtPht1–11f	catgcaccaccaacgattag
PtPht1–11r	gacettetecatgtecettg
PtPht1–12f	ggaaaccatgtgggagtgc
PtPht1–12r	ctggccagctaaagttccac
Ubiquitin f	gcagggaaacagtgaggaagg
Ubiquitin r	tggactcacgaggacag
Cys protease (CPf)	agtcactgagaaaggctgtgg
Cys protease (CPr)	ccaaatggattgttcttgctc

## **3.3.4 STATISTICAL ANALYSIS**

Identification of metabolites of interest was completed using the web-tool METAGENassist to perform principal component analysis (PCA; Arndt et al., 2012). R version 3.3.2 (R Core Team, 2016) was used to fit generalized linear models with the nlme package (Pinheiro et al., 2016), and data summaries being created using the Rmisc package (Hope, 2013). ANOVA was used to test the significance of the categorical predictors and their interactions. Posthoc multiple comparisons tests using the Tukey method were completed to test significance between sample means.

## **3.4 RESULTS**

Of the seven poplar genotypes grown in the 2014 greenhouse trial, four were selected for further analysis based on their uptake and storage potential. P39 and Walker were considered as minimal phosphate accumulators, while conversely Northwest and Tristis were considered high-accumulating genotypes, based on their leaf phosphorus content (Fig. 2.4) and the proportion of phosphorus stored as phosphate in leaf tissue (Table 3.2). Differences between the optimal nutrient ratio (100N:13P:70K) and the highest phosphate ratio (100N:195P:291K) were compared.

TABLE 3.2: UNDER OPTIMAL CONDITIONS, THE PERCENTAGE OF PHOSPHORUS STORED AS PHOSPHATE IN LEAVES.

Genotype	% PO4
P39	56.8
Walker	75.1
Northwest	80.8
Tristis	91.1

To maintain solubility of phosphate in the nutrient solution, potassium was added to the highest phosphate treatment (100N:195P) at a ratio above what is required for optimal plant growth, resulting in the need to track alterations in the cation concentration of leaves. In all genotypes, mature leaf potassium concentrations were greater under treatment. Although calcium levels in each treatment remained unchanged, Tristis had significantly higher calcium uptake (Table 3.3).

TABLE 3.3: INORGANIC CATIONS (% DRY WEIGHT) AND CATION AND ANION CONCENTRATIONS (MM) IN MATURE POPLAR LEAVES WITH ASSOCIATED SEM. CATIONS ARE THE SUMMED CONCENTRATIONS OF POTASSIUM, CALCIUM, AND MAGNESIUM LEAF CONCENTRATIONS WHILE ANIONS REPRESENT PHOSPHATE, SULFATE, MALATE, OXALATE AND CITRATE IN MM. BOLDED NUMBERS REPRESENT SIGNIFICANT DIFFERENCES (P < 0.05) between treatments.

100N:13P						
	Potassium (%)	Calcium (%)	Magnesium (%)	Cations (mM)	Anions (mM)	
P39	1.74 (0.07)	1.29 (0.11)	0.50 (0.06)	0.97 (0.07)	0.36 (0.06)	
Walker	1.67 (0.05)	0.73 (0.04)	0.32 (0.02)	0.74 (0.03)	0.44 (0.00)	
Northwest	2.14 (0.14)	1.17 (0.04)	0.50 (0.02)	1.05 (0.02)	0.50 (0.03)	
Tristis	1.39 (0.12)	0.79 (0.02)	0.32 (0.02)	0.69 (0.02)	0.43 (0.01)	
	100N:195P					
P39	<b>2.42</b> (0.16)	1.28 (0.12)	0.48 (0.03)	1.13 (0.08)	0.49 (0.04)	
Walker	<b>2.72</b> (0.31)	0.82 (0.03)	0.28 (0.01)	<b>1.01</b> (0.06)	<b>0.62</b> (0.01)	
Northwest	<b>2.96</b> (0.12)	1.24 (0.16)	0.54 (0.06)	<b>1.29</b> (0.03)	0.76 (0.07)	
Tristis	<b>3.26</b> (0.13)	<b>1.33</b> (0.10)	0.47 (0.05)	<b>1.36</b> (0.06)	<b>1.26</b> (0.03)	

## **3.4.1** INDIVIDUAL METABOLITE RESPONSE TO INCREASING PHOSPHATE STATUS

Increasing external concentrations of phosphate and the resulting accumulation in tissues had little effect on concentrations of individual metabolites. In addition to the increased concentrations of phosphate in the 100N:195P treatment, roots, leaves and senescent leaves, had altered organic acid concentrations. The most notable pattern appeared in mature leaf tissue, which showed significant increases in malate and citrate in P39, oxalate and citrate in Walker, and all three measured organic acids in Tristis. Organic acid concentrations had a high degree of variation, and as such the three organic acids were summed for each biological replicate and significance was reaffirmed. Differences in Walker and Tristis remained significant (p < 0.05) with increases in organic acid concentrations of 2.71 % and 6.78 %, respectively in mature leaves under high-phosphate treatments (Table 3.4). Mature leaf starch concentrations at high external phosphate showed a decreasing trend in P39 and Northwest, with a significant decrease in Tristis (24.2 % and 17.1 % in mature and senescent leaves, respectively; Table 3.5).

Roots				100N:13P			
		Phosphate	Sulfate	Malate	Oxalate	Citrate	Organic acids
	P39	1.07 (0.06)	0.97 (0.10)	0.64 (0.03)	1.32 (0.17)	0.31 (0.04)	2.27 (0.20)
	Walker	1.13 (0.06)	0.91 (0.13)	0.39 (0.03)	1.18 (0.04)	0.10 (0.00)	1.67 (0.01)
	Northwest	1.12 (0.05)	1.07 (0.25)	0.79 (0.25)	1.19 (0.05)	0.41 (0.05)	2.40 (0.26)
	Tristis	1.04 (0.02)	1.04 (0.11)	0.87 (0.06)	1.81 (0.11)	0.50 (0.06)	3.18 (0.18)
				100N:195P			
	P39	1.51 (0.02)	0.82 (0.09)	0.69 (0.04)	1.48 (0.07)	0.38 (0.02)	2.55 (0.08)
	Walker	<b>2.04</b> (0.13)	1.02 (0.11)	0.47 (0.08)	1.00 (0.08)	0.15 (0.02)	1.62 (0.17)
	Northwest	<b>1.73</b> (0.07)	0.73 (0.07)	0.53 (0.06)	0.97 (0.08)	0.40 (0.05)	1.89 (0.14)
	Tristis	<b>1.87</b> (0.06)	1.16 (0.16)	0.95 (0.04)	1.83 (0.08)	0.52 (0.04)	3.30 (0.13)
Mature leaves				100N:13P			
		Phosphate	Sulfate	Malate	Oxalate	Citrate	Organic acids
	P39	0.51 (0.10)	0.63 (0.09)	0.96 (0.24)	1.02 (0.12)	0.92 (0.20)	2.91 (0.49)
	Walker	0.91 (0.08)	1.79 (0.12)	0.94 (0.11)	0.76 (0.02)	0.25 (0.08)	1.95 (0.21)
	Northwest	1.21 (0.18)	0.78 (0.10)	1.87 (0.11)	0.86 (0.13)	1.05 (0.25)	3.78 (0.33)
	Tristis	1.34 (0.21)	0.87 (0.06)	0.61 (0.09)	1.21 (0.09)	0.79 (0.16)	2.61 (0.34)
				100N:195P			
	P39	0.76 (0.05)	0.66 (0.09)	1.71 (0.27)	1.12 (0.09)	<b>1.62</b> (0.09)	4.45 (0.30)
	Walker	<b>1.37</b> (0.02)	1.66 (0.11)	1.87 (0.55)	<b>0.95</b> (0.04)	<b>1.85</b> (0.45)	<b>4.66</b> (0.99)
	Northwest	<b>3.27</b> (0.08)	1.04 (0.18)	1.84 (0.56)	0.86 (0.21)	1.50 (0.53)	4.20 (1.26)
	Tristis	<b>4.75</b> (0.54)	1.23 (0.11)	<b>4.59</b> (0.74)	<b>2.20</b> (0.14)	<b>2.60</b> (0.42)	<b>9.39</b> (0.40)

TABLE 3.4: Organic acid and inorganic anion concentrations (% dry weight) of four poplar genotypes. The organic acids column is the summed values of malate, oxalate and citrate. Bolded numbers represent a significant difference (p < 0.05) between phosphate treatments.

Senescent leaves	100N:13P						
		Phosphate	Sulfate	Malate	Oxalate	Citrate	Organic acids
	P39	1.44 (0.14)	1.08 (0.09)	2.36 (0.33)	0.65 (0.19)	0.89 (0.24)	3.90 (0.57)
	Walker	3.28 (0.27)	3.39 (0.27)	1.16 (0.34)	1.02 (0.19)	0.50 (0.22)	2.68 (0.69)
	Northwest	2.17 (0.31)	0.92 (0.11)	2.30 (0.62)	1.71 (0.41)	2.00 (0.88)	6.89 (1.00)
	Tristis	3.29 (0.08)	1.34 (0.08)	1.26 (0.26)	1.93 (0.19)	1.09 (0.28)	4.28 (0.20)
				100N:195P			
	P39	<b>2.16</b> (0.15)	0.90 (0.04)	2.69 (0.33)	0.59 (0.08)	1.20 (0.25)	4.49 (0.58)
	Walker	<b>5.15</b> (0.30)	2.28 (0.24)	2.41 (0.33)	1.04 (0.11)	<b>2.36</b> (0.15)	<b>5.82</b> (0.34)
	Northwest	<b>5.41</b> (0.66)	0.96 (0.05)	1.58 (0.33)	0.86 (0.25)	0.89 (0.32)	<b>3.33</b> (0.37)
	Tristis	<b>9.50</b> (0.17)	1.70 (0.14)	1.54 (0.23)	2.74 (0.26)	2.44 (0.94)	6.72 (1.24)

Roots 100N:13P Raffinose myo-inositol Galactinol Galactose Glucose Fructose Sucrose Stachyose Starch P39 0.08 (0.01) 0.08 (0.02) 0.14(0.02)0.02 (0.00) 0.23(0.03)1.64(0.13)\_ 5.62 (1.89) \_ Walker 0.11 (0.01) 0.18 (0.08) 0.04(0.00)0.59 (0.04) 0.42 (0.05) 9.35 (3.00) 3.64 (0.21) \_ \_ Northwest 0.13(0.02)0.23(0.10)0.04(0.02)0.46(0.17)0.48 (0.21) 2.46(0.25)9.47 (3.42) \_ \_ 0.04(0.00)0.48(0.09)Tristis 0.09 (0.01) 0.03 (0.00) 0.31 (0.04) 2.92(0.17)8.69 (1.21) \_ \_ 100N:195P P39 5.46 (1.05) 0.10(0.00)0.10 (0.02) **0.01** (0.00) 0.10(0.02)0.07(0.01) 1.32(0.07)\_ \_ Walker 0.09(0.01)0.19 (0.01) **0.02** (0.00) 0.34 (0.06) **0.21** (0.02) 2.63 (0.16) 5.87 (2.60) \_ \_ Northwest 0.14(0.02)0.22 (0.02) 0.02(0.00)0.26 (0.05) 0.27 (0.07) 2.13(0.16)13.52 (1.49) \_ Tristis 0.11 (0.00) 0.11(0.02)0.03(0.00)0.28 (0.02) 0.24(0.04)2.41 (0.38) 8.46 (2.90) \_ \_ Mature leaves 100N:13P myo-inositol Galactinol Galactose Glucose Fructose Sucrose Raffinose Stachyose Starch P39 1.14(0.04)1.45 (0.09) 0.02(0.00)0.62(0.18)0.25(0.05)4.23 (0.30) 25.74 (5.80) \_ \_ Walker 0.75(0.04)0.89 (0.08) 0.06(0.01)2.47 (0.41) 0.34(0.04)4.20 (0.06) 40.83 (1.27) \_ \_ Northwest 1.13 (0.11) 1.71 (0.19) 0.02 (0.01) 0.63 (0.17) 15.08 (5.26) 0.31 (0.08) 5.64 (0.12) \_ \_ Tristis 1.35 (0.06) 1.60 (0.11) 0.02 (0.00) 0.45 (0.10) 0.16 (0.05) 6.04 (0.11) 43.61 (0.92) \_ \_ 100N:195P P39 1.14 (0.06) 1.34 (0.07) 0.01 (0.00) 0.45 (0.08) 0.27 (0.03) 4.92 (0.24) 14.59 (2.87) \_ \_ Walker 0.87 (0.10) 0.93 (0.13) **0.01** (0.00) **0.93** (0.05) 0.20 (0.01) 37.81 (2.15) 4.53 (0.29) \_ \_ Northwest 1.21 (0.25) 1.79 (0.23) 0.01 (0.00) 0.41 (0.09) 0.22 (0.05) 7.54 (2.39) 5.70 (0.83) \_ \_ Tristis 1.53 (0.14) 2.27 (0.15) 0.02 (0.01) 0.77 (0.24) 0.27 (0.07) 6.36 (0.19) **19.42** (3.23) \_ \_

TABLE 3.5: CARBOHYDRATE CONCENTRATIONS (% DRY WEIGHT) WITH ASSOCIATED SEM OF FOUR POPLAR GENOTYPES UNDER INCREASING EXTERNAL PHOSPHATE TREATMENTS. BOLDED NUMBERS REPRESENT A SIGNIFICANT DIFFERENCE (P < 0.05) between phosphate treatments.

Senescent leaves					100N	1:13P				
		myo-inositol	Galactinol	Galactose	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Starch
	P39	1.00 (0.03)	3.91 (0.08)	0.06 (0.01)	1.02 (0.08)	0.62 (0.05)	7.37 (0.85)	0.43 (0.07)	0.70 (0.08)	0.51 (0.06)
	Walker	0.65 (0.05)	3.11 (0.34)	0.22 (0.02)	3.03 (0.44)	1.55 (0.15)	6.30 (0.63)	0.25 (0.03)	0.65 (0.11)	0.31 (0.14)
	Northwest	0.55 (0.07)	2.53 (0.49)	0.06 (0.02)	0.74 (0.23)	0.47 (0.19)	3.17 (0.77)	0.11 (0.02)	0.26 (0.01)	0.50 (0.11)
	Tristis	1.07 (0.09)	2.49 (0.35)	0.05 (0.02)	0.48 (0.13)	0.18 (0.06)	4.33 (0.51)	0.26 (0.12)	0.19 (0.14)	19.65 (3.77)
					100N	:195P				
	P39	1.02 (0.10)	4.11 (0.41)	0.05 (0.00)	0.91 (0.07)	0.59 (0.08)	7.41 (0.47)	0.48 (0.05)	0.64 (0.02)	0.58 (0.10)
	Walker	0.59 (0.03)	2.34 (0.31)	<b>0.10</b> (0.02)	1.86 (0.24)	0.99 (0.13)	5.59 (0.35)	0.26 (0.05)	0.54 (0.06)	0.81 (0.13)
	Northwest	0.59 (0.08)	2.20 (0.33)	0.03 (0.00)	0.61 (0.16)	0.38 (0.12)	3.64 (0.45)	0.18 (0.02)	0.26 (0.01)	0.60 (0.09)
	Tristis	0.81 (0.02)	2.86 (0.45)	0.05 (0.01)	0.64 (0.05)	0.35 (0.06)	4.79 (0.97)	0.21 (0.02)	0.11 (0.02)	<b>2.35</b> (1.73)

#### 3.4.2 METABOLIC PROFILES OF HIGH AND LOW PHOSPHATE ACCUMULATORS

Similarities between the metabolic profiles of the genotypes varied by tissue and by treatment. Levels of sucrose, inorganic anions and organic acids were the main sources of variation in mature leaves, senescent leaves and roots. Differences in sugar alcohols and glucose concentrations also contributed to the differing metabolic profiles of mature leaves. Starch was removed from principal component analysis to improve visualization; however, results were consistent with or without the inclusion of starch.

## 3.4.2.1 LEAVES

Under optimal nutrient conditions, the metabolic profiles of mature leaves for the lowaccumulating hybrids, P39 and Walker, grouped together as did the high-accumulators Northwest and Tristis. High external phosphate concentrations caused a significant change in the mature leaves of Walker and Tristis, but not P39 or Northwest (Fig. 3.7). Senescent leaves of all genotypes were metabolically similar. Treatment of trees with phosphate led to three significantly different metabolite groupings in senescent leaves: P39, Walker with Northwest, and Tristis (Fig. 3.8).

## 3.4.2.2 ROOTS

Differences in root metabolites were observable between P39 and Tristis under optimal growth conditions and became more pronounced under high-phosphate treatments. Changes caused by higher phosphate treatments were much less apparent in roots, compared to the other tissues examined in this study (Fig. 3.9).



FIGURE 3.7: PRINCIPAL COMPONENT ANALYSIS OF METABOLITES IN POPLAR MATURE LEAF TISSUE. SHORTHAND IN THE LEGEND INCLUDES TWO CHARACTER CODE FOR THE GENOTYPE, LETTER SIGNIFYING TISSUE TYPE AND NUMBER INDICATING THE FOLD INCREASE OF APPLIED PHOSPHATE RELATIVE TO OPTIMAL LEVELS. ELLIPSES REPRESENT THE 95 % CONFIDENCE REGION.



FIGURE 3.8: PRINCIPAL COMPONENT ANALYSIS OF METABOLITES IN POPLAR SENESCENT LEAF TISSUE. SHORTHAND IN THE LEGEND INCLUDES TWO CHARACTER CODE FOR THE GENOTYPE, TWO LETTERS SIGNIFYING TISSUE TYPE AND NUMBER INDICATING THE FOLD INCREASE OF APPLIED PHOSPHATE RELATIVE TO OPTIMAL LEVELS. ELLIPSES REPRESENT THE 95 % CONFIDENCE REGION.



FIGURE 3.9: PRINCIPAL COMPONENT ANALYSIS OF METABOLITES IN POPLAR ROOT TISSUE. SHORTHAND IN THE LEGEND INCLUDES TWO CHARACTER CODE FOR THE GENOTYPE, A LETTER SIGNIFYING TISSUE TYPE AND NUMBER INDICATING THE FOLD INCREASE OF APPLIED PHOSPHATE RELATIVE TO OPTIMAL LEVELS. ELLIPSES REPRESENT THE 95 % CONFIDENCE REGION.

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#### 3.4.3 POST BUD-SET PHOSPHATE UPTAKE AND ANION RESORPTION PROFICIENCIES

Both low- and high-accumulating hybrid poplar genotypes (Tristis and P39) had continued phosphate uptake after bud–set. Phosphate concentrations in control treatment leaves with no evidence of chlorophyll degradation, six weeks after the pre–senescence harvest, were significantly increased to 3.97 and 0.88 % for Tristis and P39, respectively (Fig. 3.10). This compares to the mature-leaf phosphate concentrations in Tristis of 1.34 % and P39 concentrations of 0.51 % (Table 3.4). In terms of resorption, P39 leaf phosphate concentrations showed little variation as chlorophyll was degraded. Tristis, although differences are not significant, showed a decreasing trend in the control treatment and an increasing trend in leaf concentrations at high field levels of phosphate. The willow hybrid AAFC–5 achieved the lowest phosphate resorption proficiency by the time 60 % of chlorophyll had been degraded (Fig. 3.10).

As for the remaining anions, including sulfate and the aforementioned organic acids, minimal resorption was apparent in P39 and AAFC–5, as leaf concentrations increased along the chlorophyll gradient. Tristis demonstrated an opposite trend with no change in sulfate concentrations but a significant reduction (53.3 %) in total organic acids (Table 3.6).



FIGURE 3.10: PHOSPHATE CONCENTRATIONS (± SEM) OF LEAVES AT DIFFERENT STAGES OF CHLOROPHYLL DEGRADATION. CHLOROPHYLL LEVELS WERE CLASSIFIED INTO FOUR CATEGORIES; 100, 80, 60 AND 40 PERCENT OF MATURE LEAF LEVELS. THE TWO POPLAR HYBRIDS AND THE WILLOW HYBRID ARE TRISTIS, P39 AND AAFC-5 RESPECTIVELY.

TABLE 3.6: LEAF ANION CONCENTRATIONS WITH ASSOCIATED SEM ACROSS A CHLOROPHYLL DEGRADATION GRADIENT. BOLDED NUMBERS REPRESENT A SIGNIFICANT DIFFERENCE (P < 0.05) WHEN COMPARED TO 100 % CHLOROPHYLL CONCENTRATIONS.

	Chlorophyll (%)	Sulfate (%)	Organic anions (%)
P39	100	1.00 (0.07)	2.27 (0.68)
	80	0.86 (0.02)	3.13 (1.36)
	60	1.12 (0.08)	4.67 (1.14)
	40	<b>1.48</b> (0.12)	6.00 (1.12)
Tristis	100	1.27 (0.15)	7.63 (0.64)
	80	1.30 (0.06)	5.66 (0.47)
	60	1.14 (0.07)	<b>3.83</b> (0.42)
	40	1.17 (0.05)	<b>3.56</b> (0.32)
AAFC-5	100	2.79 (0.18)	3.49 (0.15)
	80	3.46 (0.17)	3.06 (0.19)
	60	<b>3.75</b> (0.17)	4.63 (0.84)
	40	<b>3.94</b> (0.24)	<b>5.91</b> (0.66)

## **3.4.4 EXPRESSION OF PHOSPHATE TRANSPORTERS IN HIGH AND LOW PHOSPHATE** ACCUMULATORS

Among the poplar hybrids, the highest and lowest leaf-phosphate accumulators (Tristis and P39, respectively) were selected for comparative gene–expression analysis. Of the primers employed for targeting the 12 members of Pht1 family in poplar, all provided a product from either extracted DNA or RNA from the P39 and Tristis genotypes. From the mature leaf cDNA, no product was visible for PtPht1–2 and PtPht1–6 in P39. PtPht1–5 and PtPht1–7 showed no product in Tristis. As external phosphate concentrations increased, P39 showed increased expression of PtPht1–7 and PtPht1–9. High external phosphate levels led to a marked decrease in expression of PtPht1–2, PtPht1–4, PtPht1–9 and PtPht1–12 in Tristis (Fig. 3.11). In senescent leaves, CYS, Pht1–3, 1–7, 1–9, 1–11, 1–12 were expressed in both genotypes.



FIGURE 3.11: PCR BAND INTENSITY RELATIVE TO THE 200 BP BAND ON THE LOW MOLECULAR WEIGHT LADDER AND NORMALIZED TO UBIQUITIN. EACH BAR REPRESENTS A SINGLE POOLED SAMPLE FROM THREE INDIVIDUALS. P39 AND TRISTIS CANNOT BE COMPARED DIRECTLY, AS PRIMER EFFICIENCIES IN THESE GENOTYPES HAVE NOT BEEN ESTABLISHED.



FIGURE 3.12: PRODUCTS OF SQ-PCR ON POPLAR LEAF CDNA AT 100N:13P. UBQ IS THE UBIQUITIN REFERENCE GENE AND CYS IS A CYSTEINE PROTEASE GENE THAT IS EXPRESSED IN CELLS UNDERGOING SENESCENCE.

# 3.5 **DISCUSSION**

## 3.5.1 METABOLIC VARIATIONS BETWEEN HIGH AND LOW PHOSPHATE ACCUMULATORS

As essential nutrients, in the forms of NO<sup>3-</sup>/NH<sup>4+</sup>, P<sub>i</sub> and SO4<sup>2-</sup>, enter the plant and are converted into usable forms, cells must maintain their pH and remain electrically neutral. This equilibrium is realized only in part by H<sup>+</sup> and OH<sup>-</sup> (Roberts, 2006). Mechanistic controls to compensate for fluctuations in ion content and cellular pH involves the catabolism or anabolism of organic acids that aid to increase and decrease the pH, respectively (Britto and Kronzucker, 2005). This pH regulatory mechanism was elucidated due to observations of organic acid accumulation first in maize (Zea mays L.) and barley roots with potassium salts and subsequent studies in tobacco and castor bean (Ricinus communis L.) indicating that nitrate uptake was associated with higher malate accumulation than ammonium uptake (Torii and Laties, 1966; Zioni et al., 1971; Peuke et al., 1996). Electrochemically speaking, the two forms of inorganic nitrogen should have opposing effects; however, insights provided by studies in *Limnobium stoloniferum* show nitrate and potassium uptake increased cellular pH, and phosphate and chloride reduced pH (Ullrich and Novacky, 1990). The synthesis of these studies and evidence involving PEP carboxylase in maize during excess potassium uptake (Chang and Roberts, 1992) has led to the current models of plant cellular pH regulation (Britto and Kronzucker, 2005). As the vast majority of research has focused on nitrogen and potassium assimilation, this study aimed to confirm the effects of excess phosphate on organic acid concentrations.

Contrary to the initial hypothesis, organic acid concentrations in higher phosphate treatments increased or remained constant during excess uptake and storage of phosphate in mature leaves. This suggests that in low phosphate accumulating genotypes P39 and Walker, increases in organic acid concentrations are a response to the influx of K<sup>+</sup> (Table 3.3), where pH and electrochemical balance is maintained by the synthesis and/or carboxylation of malate with subsequent transport into the vacuole. This response may be caused by the lack of fine control of K<sup>+</sup> influx through ion channels compared to the regulated uptake of phosphate through H<sup>+</sup>/P<sub>i</sub> symporters (Chang and Roberts, 1992; Hiatt, 1967). This hypothesis is supported by the observed decrease in malate:citrate ratio (3.8 to 1.0) in Walker when plants were exposed to the high nutrient treatment, as citrate is carboxylated malic acid (Table 3.4). Conversely, P39 malate:citrate ratios remained unchanged between treatments. Of the high accumulators, Northwest demonstrated no

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changes in any mature leaf metabolite or ionic concentrations other than phosphate and potassium. It is therefore assumed that increasing potassium transport was at a high enough level to counterbalance the luxury consumption of phosphate. Tristis had increased concentrations of all organic acids, which could be attributed to increased accumulation of K<sup>+</sup> and Ca<sup>2+</sup> (Table 3.3). However, under the high treatment, Tristis had an increased malate:citrate ratio (1.8 compared to the control ratio of 0.8), indicating the potential decarboxylation of citrate. The reduction in starch observed in Tristis (Table 3.5) can be ascribed to altered leaf carbon allocation caused by the increase in organic acids (2.61 mg mgDW<sup>-1</sup> to 9.39 mg mgDW<sup>-1</sup>). It is unlikely to be caused by the Pi inhibition of the key enzyme for starch synthesis (ADP–glucose pyrophosphorylase) due to intracellular compartmentalization of the enzyme in plastids and excess phosphate in the vacuole.

# 3.5.2 CONTINUED PHOSPHORUS UPTAKE AFTER BUD-SET AND ANION RESORPTION PROFICIENCIES

Mature hybrid poplar leaves from P39 and Tristis had a 1.73– and 2.96–fold increase in phosphate concentrations six weeks after bud-set. Hybrid willow, AAFC–5, did not when compared to values observed in previous studies (Fig. 3.10; Table 3.4; Fig. 2.3). Phosphorus resorption in all genotypes measured was incomplete (> 0.08 %) according to Killingbeck (1996). It is during phase 1 and 2 of senescence where phosphate remobilization occurs with phase 2 denoted primarily by chlorophyll degradation in poplar (Keskitalo et al., 2005), and this was confirmed by the comparison of phosphorus concentrations between completely senesced leaves and those harvested at 40 % leaf chlorophyll levels (Fig. 3.10; Fig. 2.5). Phase 1 of senescence had been initiated in leaves with 100 % chlorophyll, as indicated by high expression of cysteine protease in leaves from all four chlorophyll categories as determined by SQ-PCR (data not shown). Based on these findings, an appropriate time for field assessments to obtain phosphorus or phosphate proficiencies would be once 60 % of mature leaf chlorophyll has been degraded.

Of the remaining anions investigated, assuming that leaf mass decreases as senescence progresses, Tristis showed the lowest concentrations of sulfate and organic acids in senescing leaves with 40 % remaining chlorophyll. This suggests higher sulfate resorption proficiencies and efficiencies in hybrid poplar when compared to hybrid willow (Table 3.6).

#### **3.5.3 PHOSPHATE TRANSPORTER EXPRESSION IN CONTRASTING GENOTYPES**

Since the metabolic adjustments observed in the trial appear to largely be a function of K<sup>+</sup> influx rather than the accumulation of phosphate, it can be assumed that the current regulation of phosphate transporters in poplar is sufficient to prevent overaccumulation to the point where metabolic adjustment is required to prevent toxicity symptoms. This same state of regulation also appears to have a detrimental effect on phosphate resorption efficiency and proficiency in leaves, as suggested by de Campos et al. (2013) in *Acacia* and *Banksia* species. Genotypes with the strictest regulation of uptake, such as P39, therefore have little phosphate accumulation and no discernable levels of resorption. Tristis, the highest luxury consumer among the poplar, had an increased malate:citrate ratio, reduced total starch concentrations when under high-phosphate treatment, and the highest phosphorus resorption efficiency (31.7 %) of all the hybrid poplar examined. Tristis resorption proficiencies, however, were extremely poor (Table 3.4). Given the consistent and significant differences between the low-phosphate accumulator P39 and high-phosphate accumulator Tristis, expression of the Pht1 family of phosphate transporters was determined by SQ-PCR. A preliminary assessment suggests that differential expression of Pht1 may affect the storage potential of phosphate in leaves.

Of the gene products absent from P39 or Tristis (Fig. 3.11), PtPht1–2 and PtPht1–5 are low-affinity transporters, while PtPht 1–6 is considered a high-affinity phosphate transporter, as characterized by the complementation of the yeast (*Saccharomyces cerevisiae*) phosphate transporter mutant  $\Delta pho84$  (Loth-Pereda et al., 2011). Of the genes with differential expression under the high external phosphate treatments, PtPht1–2, PtPht1–9 and PtPht1–12 have been shown to have higher expression during senescence in *P. trichocarpa*, which implies a role in the remobilization of phosphate (Loth-Pereda et al., 2011). Extending this interpretation to the findings of this study, the increased expression of PtPht1–9 and the maintained expression of PtPht1–12 under high external phosphate in P39 could be resulting in remobilization of phosphate back to the roots. The decreased expression of PtPht1–2, PtPht1–9 and PtPht1–12 in Tristis may contribute to reduced remobilization of phosphate from the leaves, manifesting in excess storage occurring in leaf mesophyll cells (Fig. 3.11). The role of PtPht1–9 and PtPht1–12 in remobilization is corroborated by the presence of strong SQ-PCR bands apparent in senescent-leaf cDNA (Fig.3.12). It is unclear if PtPht1–4 expression in Tristis results in improved remobilization, as the previous study has suggested its expression to be ubiquitous throughout all tissues, undistinguishable from PtPht1–7 transcripts with its expression unaffected by senescence status (Loth-Pereda et al., 2011).

# **3.5.4** CONCLUSIONS

Based on the extreme nature of the treatment involved in this comparison, it can be expected that at field levels of soluble phosphate, the main source of variation observed in metabolite profiles, would be genotypic differences rather than the effect of treatment. Changes in poplar leaf organic acid profiles across treatments are more likely to be caused by the influx of inorganic cations ( $K^+$ ,  $Ca^{2+}$ ) than due to the luxury uptake of inorganic anions such as phosphate. When performing comparisons of nutrient concentrations and content at varying time points or across studies, care should be taken as phosphate accumulation continues after bud set in poplar genotypes. For comparisons of phosphorus resorption proficiency, samples can be taken as early as when leaf chlorophyll is at 60 % of mature leaf levels at bud set.

As movement across the plasma membrane of phosphate is reliant upon  $H^+/P_i$  symporters, identification of those specific transporters involved in remobilization is required for understanding the mechanisms behind efflux of excess phosphate from leaf tissue to prevent toxicity during growth, as well as export to the roots for storage during leaf senescence. To that end, ideal candidates for mis–regulation in poplar would appear to be PtPht1–9 and PtPht1–12.

# 3.5.5 CONTINUING RESEARCH

Using T-DNA insertion lines available for *A. thaliana* in Pht1 gene family members, phenotypes resulting from these gene knockouts may be used to assess the role of these transporters in excess phosphate accumulation and/or the redundancy of their function. This could lead to confirmation of suitable candidates for mis–regulation in poplar with respect to phloem loading of phosphate.

# 4. CONSTITUTIVE EXPRESSION OF AN EXOGENOUS PHT1 FAMILY MEMBER IN ANNUAL AND PERENNIAL SYSTEMS.

# 4.1 SUMMARY

When plants adapted to phosphorus-impoverished soils are exposed to high soil phosphate, they are largely unable to reduce uptake as internal leaf concentrations increase. Among these plants adapted to low-phosphorus conditions, those most unable to downregulate phosphate uptake often have high leaf phosphorus resorption efficiencies when grown in normal conditions. Those that have moderate uptake show lower resorption efficiencies, suggesting constitutively expressed phosphate transporters may play dual roles in phosphate uptake from the soil and resorption from mature leaves (de Campos et al., 2013).

From a phytoremediation perspective, should this relationship hold true, it could be used to improve the phosphate-storage capacity and resorption of annual and perennial plants species growing on marginal soils with the intent of limiting phosphorus entry into riparian ecosystems. Transgenic *Arabidopsis thaliana* and hybrid poplar P39 (*Populus. alba* × *Populus grandidentata*), harboring a constitutively expressed low-affinity potato phosphate transporter (35S::StPht1-1) were generated using *Agrobacterium*-mediated transformation. For both annual and perennial species, the highest expressing 35S::StPht1-1 lines were grown alongside wild-type plants and subjected to increasing phosphate applications. StPht1-1 expression in *A. thaliana* led to a reduction in biomass when grown under high-phosphate conditions and had no effect on phosphate remobilization during senescence. StPht1-1 constitutive expression in P39 resulted in increased leaf phosphate content in the highest expressing transgenic line and minimal to no effect on P resorption efficiency. Sulfate resorption showed the greatest improvement in all three transgenic lines with a 31.0 - 37.0 % increase in resorption efficiency. These results highlight the complexity of nutrient resorption mechanisms in plants.

## 4.2 INTRODUCTION

Nutrient resorption is often studied from an ecological or crop production perspective. Broad-scale ecological studies of nutrient resorption use cross-species comparisons to identify which nutrient-conservation traits are predominantly associated with phenotypic responses to the environment versus a function of phylogeny (Aerts, 1996; Killingbeck, 1996). In crop science there is a focus on identifying genes that can increase nutrient-use efficiency, which includes increased acquisition efficiency and improved remobilization (Han et al., 2015; Veneklaas et al., 2012). Nutrient resorption has been accepted as a widely variable trait that can be affected by an array of determinants, such as age, source-sink interactions, leaching and timing of abscission, depending on the nutrient in question. However, for phosphorus in particular, none of the aforementioned variables have been shown to be correlated to resorption, leaving the controls on phosphorus resorption largely unknown (Chapin and Moilanen, 1991; Killingbeck et al., 1990).

Barriers to phosphorus remobilization are dependent upon where the largest phosphorus storage pools are located in the cell. Phosphorus in photosynthetic tissues at or above optimal crop concentrations  $(2 - 15 \text{ mg P g}^{-1} \text{ DW})$  is stored primarily in the vacuole, followed by nucleic acids, lipids and other ester phosphates (Veneklaas et al., 2012). From experiments done in barley (Mimura et al., 1990), soybeans (Lauer et al., 1989), sycamore (Acer pseudoplatanus L.) and A. thaliana (Pratt et al., 2009), it is apparent that the vacuole stores excess cellular phosphate to buffer cytoplasmic phosphate concentration and sustain cell metabolism in low phosphate conditions. The perceived mobility of vacuolar phosphate, and the assumption that sub-optimal soil phosphate results in low vacuolar phosphate, led much of the research focus to center around the release of phosphate from the organic phosphorus pool. This involved isolating enzymes required for the hydrolysis for nucleic acids and other phosphate esters, such as RNases and purple acid phosphatases (PAPs). Genes such as AtPAP26 in Arabidopsis were shown to be crucial for efficient phosphorus remobilization and the progression of senescence (Robinson et al., 2012). Once inorganic phosphate is present in the cytosol, the final barrier to transport is the plasma membrane and subsequent loading into the phloem. From previous experiments in poplar (Table 3.4), inorganic phosphate concentrations remain high in senesced leaves suggesting a lack of sufficient export from the cell was a primary barrier to resorption. The possibility of increasing phosphate export from the cell via overexpression of a Pht1 transporter was therefore explored.

Members of the Pht1 phosphate transporter family are H<sup>+</sup>/H<sub>2</sub>PO<sub>4</sub><sup>-</sup> symporters that span the plasma membrane and show high protein sequence homology both between the different members and across species (Nussaume et al., 2011). They are considered high-affinity transporters whose primary function is the uptake of phosphate from the soil matrix; however, further studies have shown a range of affinities, revealing their probable roles in phosphate translocation during active growth (Nagarajan et al., 2011; Shin et al., 2004). Transporters of particular interest that have been implicated in phosphate translocation in Arabidopsis include AtPht1-1, AtPht1-4 and AtPht1-5. AtPht1-1 and AtPht 1-4 are the most highly expressed high-affinity Pht1 transporters in roots, with low expression in aerial portions of the plant. AtPht1-4 is more widely expressed, with detectable transcripts in the root central cylinder, anthers and in floral and silique abscission zones, all which do not show expression of AtPht1-1 (Nussaume et al., 2011). AtPht1-1 is the most characterized with a measured K<sub>m</sub> of 3.1 µM, and a significant role in phosphate uptake and translocation under high external phosphate conditions (Mitsukawa et al., 1997; Shin et al., 2004). AtPht1-4 was also shown to play a significant role in phosphate uptake and translocation in conjunction with AtPht1-1; however, AtPht1-1 is able to compensate to such an extent that no phenotype is visible in AtPht1-4 insertional mutants (Shin et al., 2004). Conversely, AtPht1-5 has very weak expression in roots and high expression in older leaves at the start of senescence (Mudge et al., 2002). Twelveday-old AtPht1-5 knock-out mutant seedlings also show increased shoot and lower root phosphorus content, suggesting reduced remobilization from leaves (Nagarajan et al., 2011). Of the three Arabidopsis phosphate transporters, AtPht1-5 is the most likely to play a role in phosphate remobilization from leaves during senescence.

Based on a comparative study of two plant species native to Australia, a current hypothesis addressing controls on phosphorus resorption is that constitutively expressed low-affinity phosphate transporters play dual roles in phosphate uptake and resorption (de Campos et al., 2013). AtPht1-5 was not found to be ideal to test this hypothesis. The affinity of AtPht1-5 is unknown and its expression is strictly regulated, with overexpression leading to premature senescence (Nagarajan et al., 2011). This led to StPht1-1, a low-affinity phosphate transporter from potato, being chosen for overexpression in the hybrid poplar P39 to improve plant performance in excess phosphorus conditions. StPht1-1 has near ubiquitous expression in tubers, roots, mature leaves, petioles and flowers and a suggested  $K_m$  of 280µM, making it a putative low affinity transporter potentially participating in both uptake and translocation (Leggewie et al., 1997; Winter et al.,

2007). An AtPht1-5 insertional mutant was concurrently screened for resorption phenotypes for subsequent complementation with the potato phosphate transporter.

# 4.3 MATERIALS AND METHODS

## 4.3.1 CONFIRMATION OF T-DNA INSERTIONAL MUTANTS

Seeds of a putative T–DNA insertional line affecting AT2G32830 (AtPht1-5) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003). The line has been annotated as SALK\_106359.51.45.X. Seeds were planted directly onto soil and grown at a light intensity of 175 µmol m<sup>-2</sup>s<sup>-1</sup> for 16 hour days at 22 °C and 8 hour night cycles at 20 °C. Once plants had approximately five unfurled true leaves, genomic DNA was extracted from a single leaf for PCR confirmation of the presence of the T-DNA insertion. DNA extractions were completed by grinding leaf tissue in 0.4 mL of a buffer containing 200 mM Tris-HCl, 250 mM NaCl, 25 mM Na-EDTA, and 0.5 % SDS. Samples were centrifuged to pellet the insoluble plant material and 0.3 mL of supernatant was transferred to a new micro-centrifuge tube prior to the addition of 0.3 mL of isopropanol. DNA was precipitated via centrifugation and washed with 0.5 mL of 70 % ethanol prior to resuspension in 0.1 mL of water. Primers used for the subsequent genotyping can be found in Table 4.1. After lines were confirmed, homozygous for each T-DNA insertion, seeds were collected and the two lines were screened for discernible growth phenotypes, according to Bolle (2009).

Primer name	Sequence
SALK_106359C LP	5' – TCGGGTCTGGTCTATCATTTG – 3'
SALK_106359C RP	5' – GACAAGTCGATCTCAACCTCG – 3'
SALK_LB Primer	5' – ATTTTGCCGATTTCGGAAC – 3'

TABLE 4.1: PRIMERS	USED FOR	GENOTYPING .	ARABIDOPSIS '	Γ–DNA	<b>INSERTION LI</b>	INES

## 4.3.2 **PREPARATION OF TRANSGENIC LINES**

## 4.3.2.1 CLONING

Due to the absence of introns in the gene encoding StPht1-1, the low-affinity potato phosphate transporter was cloned directly from genomic DNA. Potato DNA was isolated using a CTAB extraction method (Ghislain et al., 1999) from the leaf tissue of Solanum tuberosum cv. Russet. A blunt-end PCR product of the StPht1-1 gene was produced using a forward primer with the required 5' CACC overhang for Directional TOPO® cloning into the pENTR Gateway® entry vector (Table 4.2; Appendix A). The resulting pENTR-StPht1-1 plasmid was transformed into chemically competent cells of the DH5a strain of Escherichia coli (Thermo Fisher Scientific, 2012). After selection of positive colonies and isolation of the pENTR–StPht1-1 plasmids, three LR recombination reactions were completed to insert the cloned StPht1-1 into multiple destination vectors (Thermo Fisher Scientific, 2003). Two destination vectors were for use in A. thaliana (pH7WGY2 and pH7YWG2) and one destination vector was for use in the P39 poplar hybrid (pK2GW7; Appendix A). The pH7WGY2 plasmid contains an N-terminal yellow fluorescent protein (YFP) tag, while the pH7YWG2 has a C-terminal YFP tag. pK2GW7 does not contain a sequence for a fluorescent protein tag. An alternate reverse primer was used to remove the StPht1-1 stop codon prior to cloning into the pH7YWG2 destination vector so the transcription of the YFP tag would not be impeded (Table 4.2). In two separate transformation events, the destination vectors pH7WGY2 and pH7YWG2 were transformed into DH5a and subsequently into Agrobacterium tumefaciens strain GV3101. pK2GW7 was transformed into the A. tumefaciens strain EHA101. Positive colonies were selected using the primers in Table 4.3.

Primer name	Sequence
Cloning-StPht1-1f	5' – CACCATGGCGAACGATTTG – 3'
Cloning-StPht1-1r (Stop Codon)	5' – TTAAACAGGAACTGTCCTTCCAC – 3'
Cloning-StPht1-1r (No Stop Codon)	5' – AACAGGAACTGTCCTTCCAC – 3'

TABLE 4.2: PRIMERS USED FOR THE CLONING OF STPHT1-1 FROM POTATO GENOMIC DNA.

TABLE 4.3: PRIMER PAIL	R USED TO VERIFY T	HE INSERTION OF STPHT1-1.
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Primer name	Sequence	
CStPht1-1f	5' – GATGCTCTTGCTTCAACAG – 3'	
T35Sr	5' – CCCTTATCTGGGAACTACTCAC – 3'	

#### 4.3.2.2 PLANT TRANSFORMATIONS

#### 4.3.2.2.1 ARABIDOPSIS

Wild-type A. thaliana ecotype 'Columbia' (Col-0) seeds were sterilized and plated onto 0.5x MS media before being stratified at 4 °C for two days. After two weeks of growth under longday conditions (16 hour photoperiod at 22 °C, seedlings were transferred to wet soil with four seedlings per pot. Two trays of Col-0 were planted and placed into a growth chamber under longday conditions. Day temperature was 22  $^{\circ}\mathrm{C}$  with a light intensity of 175  $\mu mol~m^{\text{-2}}s^{\text{-1}}$  and night temperatures were 20 °C. For the first week in the growth chamber, seedlings were covered in opaque plastic to maintain high humidity. Plants were grown under these conditions until bolting and flowering began. As flowering began, a 5 mL liquid culture of A. tumefaciens strain GV3101 containing pH7WGY2-StPht1-1 and a 5 mL culture of GV3101 containing pH7YWG2-StPht1-1 were prepared and grown at 28 °C for two days. After two days, each feeder colony was used to inoculate 100 mL of liquid LB media in a 500-mL flask and grown at 28 °C at 180 rpm for 20 hours or until the colonies had an OD of 1.5 - 2.0. The cultures were then poured into their separate 50-mL Falcon tubes and centrifuged at 4000 g for 10 minutes before being resuspended in an equal volume of 5 % (wt/vol) sucrose solution. Sylgard 309 was added to each volume to a concentration of 0.02 % (vol/vol) and mixed well. The resulting culture solution was poured into a beaker into which A. thaliana plants were inverted and the entire inflorescence submerged for 10 seconds. Plants were allowed to drain when removed from the cell suspension before being placed back onto the tray. One tray of plants was dipped per construct for a total of 72 plants per construct. Once a tray was processed, plants were laid horizontally and covered in a black plastic bag for 24 hours. Plants were dipped again with the same construct one week later to achieve a higher frequency of transformation. After removal of the black plastic, plants were grown in the growth chamber until seeds had set. Seeds were collected and stored at room temperature. Transformed

lines were selected by sterilizing seed and plating them onto 0.5x MS media containing carbenicillin and hygromycin. Seedlings that produced true leaves were transferred to soil and the insertion of StPht1-1 was confirmed using the primers in Table 4.3. Plants with PCR-confirmed insertion of StPht1-1 were grown for successive generations until homozygous lines were attained. The entire transformation and selection protocol was adapted from Zhang et al. (2006). Presence of the StPht1-1 protein was confirmed by visualizing the YFP tag in four-day-old hypocotyl cells on the confocal microscope. Microscope settings were as follows: 20x objective lens ( $\Sigma$  200x), INT5 AP9, exposure of 990 ms, sensitivity 161, intensity 51 %.

## 4.3.2.2.2 HYBRID POPLAR

The poplar hybrid P39 (*P. grandidentata*  $\times$  *P. alba*) was clonally propagated in woody plant medium (WPM) (McCown and Lloyd, 1981) using plant tissue culture techniques. After plants were grown for 4-5 weeks, leaf discs were cut from the top three leaves of each plant, avoiding the mid-vein, and stored overnight in batches of 25 leaf discs in liquid WPM. At the same time, the A. tumefaciens strain EHA101 with the inserted pK2GW7-StPht1-1 construct was grown overnight in 90 mL of liquid WPM with 2 % sucrose and 900 µL of a 20 mM acetosyringone solution. The following day, the cell culture was diluted using liquid WPM to an OD of 0.1 - 0.2. From each batch of 25 leaf disks, the liquid WPM was drained and replaced with ~10 mL of the cell culture solution. Leaf disks and the A. tumefaciens solution were co-cultivated in darkness for at least 30 minutes at 20 °C and 85 rpm. After which, leaf disks were blotted dry and plated adaxial side down onto solid WPM media supplemented with 0.1  $\mu$ M  $\alpha$ -naphthalene acetic acid (NAA),  $0.1 \,\mu\text{M}$  6-benzylaminopurine (BA) and  $0.1 \,\mu\text{M}$  thidiazuron (TDZ). Plates were left in the dark, at room temperature, for three days before transferring the leaf disks to solid WPM media with 0.1  $\mu$ M of NAA, BA and TDZ, as well as 500  $\mu$ g mL<sup>-1</sup> of carbenicillin and 250  $\mu$ g mL<sup>-1</sup> of cefotaxime to kill the A. tumefaciens remaining on the leaf disks. Plates were incubated in the dark at room temperature for three more days before again transferring to fresh plates supplemented with all three hormones, carbenicillin, cefotaxime and 25 µg mL<sup>-1</sup> of kanamycin to select for plant cells successfully harbouring the pK2GW7- StPht1-1 construct. These plates were then left to grow under low light conditions for four weeks. After four weeks, shoots growing from each leaf disk were visible and one shoot per leaf disk was subsequently excised and placed onto solid WPM plates with 0.01  $\mu$ M BA, 500  $\mu$ g/mL of carbenicillin and 250  $\mu$ g mL<sup>-1</sup> of cefotaxime and 25  $\mu$ g

mL<sup>-1</sup> of kanamycin. After a further four weeks of growth, elongated shoots were transferred to WPM plates containing 0.01  $\mu$ M of NAA and the three aforementioned antibiotics. The elongated shoots rooted after four weeks and cuttings were planted into WPM containing 0.01  $\mu$ M of NAA and 25  $\mu$ g mL<sup>-1</sup> of kanamycin. Plants which grew and rooted well had cuttings taken and planted onto WPM with 0.01  $\mu$ M of NAA and 50  $\mu$ g mL<sup>-1</sup> of kanamycin. The transformation protocol was adapted from Coleman et al. (2006). Cuttings which grew successfully on the higher concentration of kanamycin were genotyped as described in section 4.3.1 and the insertion of StPht1-1 was confirmed by PCR. Plants with successful insertions were given individual identifiers and underwent further screening to select those with the highest StPht1-1 transcript abundance. The 12 plants are hereafter referred to as lines.

## 4.3.2.2.3 LINE SELECTION

For all 12 poplar lines and three homozygous A. thaliana pH7YWG2-StPht1-1 lines with visible YFP tags, RNA was extracted from leaf tissue using the TRIzol<sup>TM</sup> reagent and protocol from Thermo Fisher Scientific (2013) to measure StPht1-1 transcript abundance. An overview of the protocol is as follows: 1 mL of TRIzol<sup>TM</sup> reagent was added to 100 mg of frozen and ground leaf tissue followed by a 5 minute incubation at room temperature. 0.2 mL of chloroform was then added and the tube inverted before incubating for an additional 2 - 3 minutes. Samples were centrifuged for 15 minutes at 12000 g at 4 °C and the upper aqueous phase transferred to a new micro-centrifuge tube. 0.5 mL of cold isopropanol was added to the aqueous phase and incubated at room temperature for 10 minutes. The sample was again centrifuged for 10 minutes at 12000 g and 4 °C. The supernatant was discarded and the pellet washed in 1 mL of 75 % ethanol. A final centrifuge at 7500 g at 4 °C for 5 minutes was done before drying the pellet for 15 minutes. RNA pellets were resuspended in 20 µL of RNA-free water (Thermo Fisher Scientific, 2013). Then, a DNase clean-up and cDNA synthesis was completed as described in Section 3.3.3. The resulting cDNA was diluted to 1/10 of the original concentration. The final 10 µL qPCR reaction included SsoFast<sup>TM</sup> EvaGreen<sup>®</sup> Supermix, reference or target gene qPCR forward and reverse primers (Table 4.4), and 1 µL of diluted cDNA. Serial dilutions were performed to calculate the primer efficiencies of the reference genes (UBQ and TIF) and the target gene (StPht1-1). UBQ was chosen as the reference gene as the primer efficiency was closest to that of StPht1-1. qPCR reactions were completed on the Bio-Rad CFX96<sup>TM</sup> real-time PCR system following the Bio-Rad optimized

cycling conditions stated for cDNA with an annealing/extension step at 55 °C for 5 seconds (Bio-Rad, 2016). Three *A. thaliana* lines were used in subsequent growth chamber experiments, while the three poplar lines with the highest StPht1-1 transcript abundance were bulked on WPM, 0.01  $\mu$ M NAA media until numbers were sufficient for greenhouse experiments.

Primer name	Sequence
CStPht1-1f	5' – GATGCTCTTGCTTCAACAG – 3'
CStPht1-1r	5' – CTTGCAACACCTTGGACATG – 3'
PtUBQf	5' – GTTGATTTTTGCTGGGAAGC – 3'
PtUBQr	5' – GATCTTGGCCTTCACGTTGT – 3'
AtUBQf	5' – CAGCTCCACAGGTTGCGTTA – 3'
AtUBQr	5' – CAAGCCGAAGAAGATCAAGCACAAG – 3'

#### TABLE 4.4: PRIMERS USED FOR QPCR

## 4.3.3 **GROWTH TRIALS**

#### 4.3.3.1 ARABIDOPSIS

For all *A. thaliana* growth trials, seeds were surface-sterilized with 70 % ethanol and 10 % bleach then left to stratify in the dark at 4 °C for two days. After two days, seeds were planted directly onto soil with four plants per pot in a randomized block design within a tray. One tray was subjected to a 0.1x MS with no phosphorus, while a second tray was given 0.1x MS with 20x regular phosphorus amounts. Treatments were applied to T-DNA lines once every week for four weeks. The treatment regime was adjusted for the transformed lines, where all watering was done using the treatment solution. Plants were grown under a light intensity of 175  $\mu$ mol m<sup>-2</sup>s<sup>-1</sup> with 16 hour days at 22 °C and 8 hour night at 20 °C. On the fourth week, before plants began to bolt, two plants from every pot were harvested and lyophilized. Dry weights of the plant were taken before the plant was ground using a FastPrep machine for 20 – 40 seconds at speed setting 4.0. 10 mg of dried tissue was then weighed and acid-extracted so phosphate and sulfate levels could be determined (Section 3.3.2.2). The remaining plants were then watered as needed until all were

fully senesced. Once senesced, rosettes were collected and lyophilized before dry weight was taken and the same acid extraction performed.

For T-DNA insertional lines, extra plants were grown and total RNA was extracted using the TRIzol<sup>TM</sup> method (Section 4.3.2.2.3) to confirm the lack of transcript in the homozygous lines. Wild-type controls failed to produce bands in both leaf and root tissue. Seeds from each line and wild-type were therefore plated onto 0.5x MS plates with  $10 \,\mu\text{M}\,\text{H}_2\text{PO}_4^-$  and grown for five weeks before extracting RNA from the accumulated root tissue. PCR was completed on the resulting RNA to test presence/absence of transcript.

# 4.3.3.2 HYBRID POPLAR

Two separate trials were conducted to determine the effects of StPht1-1 overexpression on the accumulation and resorption of phosphate and sulfate in hybrid poplar. During the first trial, which ran from November 2016 – March 2017, the three lines with the highest transcript abundance were grown with appropriate wild-type trees in a randomized block design and exposed to three fertilizer treatments with the following nutrient ratios: 100N:0P, 100N:13P, 100N:195P. The ninth leaf was harvested from the trees after 10 weeks of growth and again when the leaves were  $\sim 60 \%$  yellow. Leaves were lyophilized for 20 – 24 hours and ground for phosphate and sulfate determination via HPLC (Section 3.3.2.2). Samples from leaves and roots were also taken to confirm StPht1-1 expression in both tissues. The second trial, which ran from August – December 2017, was a repeat of the first except trees were exposed to only two treatments, 100N:13P and 100N:195P. The intent of the second trial was to get phosphate and sulfate data for roots and leaves before and after leaf senescence, as well as achieve a better leaf resorption measurement by sampling after leaf abscission.

#### 4.3.4 STATISTICAL ANALYSIS

R version 3.3.2 (R Core Team, 2016) was used to fit linear mixed–effects models with the packages lmerTest (Kuznetsova, 2016) and nlme (Pinheiro et al., 2016). ANOVA was used to compare various models, and to test the significance of the categorical predictors and their interactions. Multiple comparisons were done using the Tukey method as a post-hoc test to identify significance between sample means.

# 4.4 **RESULTS**

## 4.4.1 PHT 1 T-DNA INSERTION MUTANTS

## 4.4.1.1 CONFIRMATION OF KNOCKOUT LINES

The Atpht1-5 Salk line was known to have a T-DNA insertion located in an exon (http://signal.salk.edu/cgi-bin/tdnaexpress). PCR confirmation of the genotypes showed that lines lacked full-length wild-type bands, while amplifying shorter length T-DNA primer products (data not shown). RNA extractions completed on several tissues to confirm a lack of transcript. Wild-type controls failed to produce bands for AtPht1-5 in both leaf and root tissue from plants grown in soil, on 0.5x MS agar and 0.5x MS agar with 25  $\mu$ M H<sub>2</sub>PO<sub>4</sub><sup>-</sup>. From root tissue of plants grown on 10  $\mu$ M H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, wild-type Pht1-5 bands were produced with no corresponding bands present in the Atpht1-5 homozygous Salk lines.

## 4.4.1.2 INSERTIONAL MUTANT PHENOTYPES

No differences in growth were observed between Atpht1-5 and wild-type plants grown on agar plates, soil, or when exposed to optimal and excessive phosphorus conditions. Determination of phosphate concentrations in mature leaves, senescent leaves and seeds also failed to demonstrate observable differences between wildtype and the T-DNA line. Overall, mature leaves, senescent leaves and seeds under phosphorus replete conditions (20P) contained 0.41, 0.38 and 0.85 %P, respectively (Fig. 4.1). The majority of phosphorus in seeds was present as phytic acid, while all phosphorus detected in leaf tissue was present as inorganic phosphate.



FIGURE 4.1: PHOSPHORUS CONCENTRATIONS (± SEM) AS CALCULATED FROM PHOSPHATE AND PHYTIC ACID IN VARIOUS A. THALIANA PLANT PARTS BEFORE AND AFTER SENESCENCE IN WILD-TYPE AND ATPHT1-5. ASIDE FROM THE NUTRIENTS IN THE SOIL MIX, PLANTS WERE GIVEN 0.1X MS DEVOID OF PHOSPHORUS (0P) OR 0.1X MS WITH 20 TIMES THE USUAL PHOSPHATE CONCENTRATIONS (20P).

#### 4.4.2 INTERNAL PHOSPHORUS CONCENTRATIONS IN WILD-TYPE ARABIDOPSIS AND P39

## 4.4.2.1 ARABIDOPSIS

Data collected on wild-type plants during the screening of the T-DNA insertional lines showed that differing phosphate treatments did not result in altered leaf phosphorus concentrations. Overall the average concentration was 0.45 %P. Differences arose when comparing mature to senescent leaves, with significantly lower phosphorus concentrations (p < 0.05) occurring in senescent leaves, only when no additional phosphate was provided to the plants (Fig. 4.1).

## 4.4.2.2 HYBRID POPLAR

As determined in previous experiments, wild-type P39 had significantly higher leaf phosphate concentrations under 15x optimal phosphorus treatments, increasing from 0.51 to 0.76 %Pi. Senescent leaves had higher concentrations still, with samples from optimal and excessive phosphate treatments containing 1.44 and 2.16 % Pi, respectively (Fig. 4.2).



Figure 4.2: Mean phosphate concentration ( $\pm$  SEM) in P39 leaf tissue when exposed to two levels of external phosphate, before (left) and after (right) senescence (n = 4). Asterisks (\*) indicate p < 0.05.

## 4.4.3 STPHT1-1 TRANSCRIPT LEVELS IN TRANSGENIC LINES

## 4.4.3.1 ARABIDOPSIS

Visualization of the YFP-tagged StPht1-1 protein in the elongated hypocotyls of three *A*. *thaliana* lines showed the protein localized to the plasma membrane (Fig. 4.3). Transcript abundance in leaves was determined and expression relative to ubiquitin was 0.33, 0.75 and 1.38 for lines 1, 2 and 3, respectively (Fig. 4.4). Semi-quantitative PCR performed on root RNA using StPht1-1 primers produced bright bands in all three lines and was absent from wild-type controls (data not shown).



FIGURE 4.3: VISUALIZATION OF YFP-TAG ON PLASMA MEMBRANE BOUND STPHT1-1 PROTEIN IN THE ETIOLATED HYPOCOTYLS OF A. *THALIANA* TRANSGENIC LINES.

#### 4.4.3.2 HYBRID POPLAR

StPht1-1 transcript abundance was analyzed in 12 different lines of P39 harbouring the 35S::StPht1-1 insert. Three lines displaying the highest expression were selected for use in subsequent growth trials and were designated as lines 1, 2 and 3. Relative expression, using ubiquitin as the reference gene, was 0.20, 0.31 and 0.90 for lines 1, 2, and 3 respectively (Fig. 4.4). Semi-quantitative PCR with StPht1-1 primers on root tissue collected from greenhouse-grown trees produced bright bands in all three lines with no band appearing in the wild-type control trees (data not shown).


FIGURE 4.4: RELATIVE EXPRESSION ( $\pm$  SEM) OF STPHT1-1 USING UBIQUITIN AS THE REFERENCE GENE IN BOTH A. THALIANA AND P39. A) EXPRESSION OF STPHT1-1 IN THREE HIGHEST EXPRESSING TRANSGENIC LINES OF A. THALIANA VAR. COLUMBIA (N = 3). B) EXPRESSION OF STPHT1-1 IN THE THREE HIGHEST EXPRESSING TRANSGENIC LINES OF POPLAR HYBRID P39 (N = 3).

## 4.4.4 PHOSPHATE UPTAKE AND RESORPTION IN TRANSGENIC LINES WITH OVEREXPRESSION OF STPHT1-1

#### 4.4.4.1 ARABIDOPSIS

Under phosphorus-sufficient conditions, phosphate content between the wild-type and transgenic *A. thaliana* lines did not differ. Only under excess phosphate conditions did differences appear between wild-type and line 3 plants (Table 4.1). Line 3 had significantly lower phosphate content largely due to lower biomass production rather than a decrease in internal phosphate concentrations. Sulfate content across treatments and phosphate/sulfate resorption efficiencies were unaffected by the insertion of the StPht1-1 gene.

TABLE 4.1: PHOSPHATE AND SULFATE CONTENT IN TRANSGENIC *Arabidopsis* plants. Asterisks (\*) indicate a significant difference (p < 0.05) when comparing transgenic lines to wild-type.

Mature leaves			Senescent leaves		
Sufficient P			Sufficient P		
Line	Phosphate content (mg)	Sulfate content (mg)	Line	Phosphate content (mg)	Sulfate content (mg)
Wild type	0.39	0.12	Wild type	0.17	0.01
Line 1	0.74	0.28	Line 1	0.16	0.01
Line 2	0.33	0.13	Line 2	0.17	0.02
Line 3	0.35	0.22	Line 3	0.22	0.01
Mature leaves			Senescent leaves		
Excess P			Excess P		
Line	Phosphate content (mg)	Sulfate content (mg)	Line	Phosphate content (mg)	Sulfate content (mg)
Wild type	1.24	0.23	Wild type	1.09	0.03
Line 1	1.03	0.18	Line 1	0.71	0.01
Line 2	0.90	0.15	Line 2	0.77	0.02
Line 3	0.71*	0.24	Line 3	0.86	0.01

### 4.4.4.2 HYBRID POPLAR

Phosphate content in mature leaves of hybrid poplar did not differ when trees were treated with 100N:0P and 100N:13P nutrient solutions. When exposed to 15x optimal level of phosphorus (100N:195P), wild type, line 1, and line 2 accumulated 1.7x more phosphate in a mature leaf. At 100N:195P, line 3 had a 2.1-fold increase in phosphate content and accumulated a significantly higher amount of phosphorus than wild type and the other two lower-expressing transgenic lines

(Fig. 4.5). Roots did not attain higher internal concentration of phosphorus in transgenic lines versus wild-type trees (data not shown).

For an assessment of resorption efficiencies, phosphate content in mature and senescent leaves were compared. Wild-type trees showed significantly higher content in senescent leaves, while all transgenic lines had similar values for mature and senescent leaf content (Fig.4.6A) Interestingly, transgenic lines 1, 2 and 3 had 31.3 %, 32.3 % and 36.8 % sulfate resorption efficiencies respectively, while wild-type trees did not show significant resorption of sulfate (Fig. 4.6B). An assessment of mature leaves showed an average chlorophyll concentration index (CCI) of 43.1, while all senescent leaves had a CCI < 5.0.



FIGURE 4.5: MEAN PHOSPHATE CONTENT ( $\pm$  SEM) OF P39 pre-senescent leaves when exposed to three phosphate treatments (n = 4). 1, 2 and 3 represent the three lines with highest StPht1-1 expression. Asterisks (\*) indicate p < 0.05 when compared against wild-type trees (Wt).



Figure 4.6: A comparison of phosphate and sulfate content in wild-type P39 and each of the three transgenic lines. A) Mean phosphate content ( $\pm$  SEM) of mature leaves and senescent leaves at the control treatment of 100N:13P (n = 4). B) Mean sulfate content ( $\pm$  SEM) of mature leaves and senescent leaves and senescent leaves. Phosphate treatments did not affect sulfate content resulting in the pooling of biological replicates across treatments (n = 10). Asterisks (\*) indicate p < 0.05.

#### 4.5 **DISCUSSION**

## 4.5.1 ROLES OF THE ARABIDOPSIS PHT1 FAMILY OF H<sup>+</sup>/H<sub>2</sub>PO<sub>4</sub><sup>-</sup> SYMPORTERS IN PHOSPHATE REMOBILIZATION.

The lack of phenotype in Atpht1-5 lines was unexpected as a previous study showed that Atpht1-5 mutants have a higher shoot phosphorus content under phosphate-replete conditions (Nagarajan et al., 2011). Overexpression of AtPht1-5 also resulted in increased phosphate accumulation in siliques and premature senescence, implying that AtPht1-5 plays a large role in phosphate remobilization from source to sink tissues (Nagarajan et al., 2011). This effect on remobilization is refuted by the data shown in Fig. 4.1 where an Atpht1-5 knock-out mutant, with confirmed lack of transcript, did not result in a higher senescent leaf phosphorus concentration or reduced phosphorus accumulation in seeds. This is assumed to be due to the redundant function of phosphate transporters in *Arabidopsis*, which has already been shown to occur extensively in roots (Ayadi et al., 2015). The full extent of redundancy in transporters involved in phosphate transporters such as PHO1 (Stigter and Plaxton, 2015). To definitively link AtPht1-5 transporters to remobilization during senescence, phenotypic lines would need to be isolated and complemented up to the initiation of senescence to avoid confounding growth phenotypes.

## 4.5.2 SUITABILITY OF WILD-TYPE ARABIDOPSIS AND P39 FOR THE CONSTITUTIVE EXPRESSION OF STPHT1-1

Arabidopsis thaliana var. Columbia and the poplar hybrid P39 were suitable candidates in which to express StPht1-1 for a variety of factors. Under sufficient external phosphorus conditions, both species had internal phosphate levels of ~ 0.4 %P, which is consistent with values common to crop plants grown under optimal conditions (Marschner, 2012). When exposed to weekly treatments of excess phosphate, *A. thaliana* showed no increase in cellular phosphate concentrations, while the hybrid poplar trees showed small but significant increases in phosphate concentration, meaning that they have little propensity for accumulating excess phosphate. Poor resorption proficiencies were observed in both species, well above the threshold of 0.05 %P (Killingbeck, 1996) (Fig.4.1; Fig. 4.2). This was suitable for constitutive expression of a low-affinity phosphate transporter, as their low resorption and observed status as low accumulators allowed for gains in both parameters to be assessed.

## 4.5.3 EFFECTS OF CONSTITUTIVE EXPRESSION OF AN EXOGENOUS PHT1 MEMBER ON UPTAKE AND RESORPTION

Nutrient remobilization and senescence are both complex events that are initiated by many environmental and metabolic factors. As a consequence, very little is known about the mechanisms regulating the efflux of anions, such as sulfate and phosphate, from the cell (Himelblau and Amasino, 2001; Smith et al., 2000). An inherent difficulty in the study of senescence is that genes involved in nutrient transport, likely play a role in plant growth and development. This means that knockout mutants could exhibit drastic growth phenotypes, masking the effects on senescence. Proof of function in senescence would therefore require mutants to be complemented up to the initiation of senescence. Redundancy may also be an issue, with the potential for multiple gene knockouts being required to produce a discernable phenotype (Himelblau and Amasino, 2001). This is not currently feasible in high-utility species such as poplar, due to their long life spans, but may be realized in the near future with the advent of CRISPR/Cas9 as a genome-editing tool in poplar (Zhou et al., 2015). To circumvent these difficulties, we chose to address a hypothesis put forward by de Campos et al. (2013), who suggested that constitutively expressed transporters could play dual-roles in uptake and resorption. A well-characterized low-affinity transporter was chosen to avoid the potentially toxic accumulation of phosphorus brought on by the overexpression of high-affinity transporters in roots (Smith et al., 2000).

Constitutive, plant-wide expression of StPht1-1 had different effects in the plant species used in this study. In the annual *A. thaliana*, plants expressing StPht1-1 and grown under high-phosphorus treatments had decreasing phosphate content across the transgenic lines with a significant decrease in content occurring in the highest expressing line (Table 4.1). This was caused by reductions in biomass rather than concentration, suggesting accumulation to the point of toxicity (Teng and Timmer, 1990). As for resorption, only treatment affected phosphate resorption efficiency, supporting the hypothesis that sink strength is a driver of phosphorus resorption in *A. thaliana* (Table 4.1). Overall, expression of StPht1-1 did not increase the performance of *A. thaliana* in excess phosphate conditions.

In the perennial species P39, transgenic lines expressing StPht1-1 displayed an increase in leaf phosphate content when trees were exposed to high levels of external phosphate (Fig. 4.5). This suggests that constitutive expression of an exogenous low-affinity Pht1 transporter can reduce

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the ability of hybrid poplar to moderate phosphate uptake when external phosphate concentrations are high, making it a positive modification for the use of hybrid poplar in phytoremediation applications. No measurable differences in biomass were observed between lines, indicating that no toxicity symptoms were induced by the increased accumulation of phosphate (Teng and Timmer, 1990). A positive effect on resorption was seen in all three transgenic lines compared to wild-type trees, both in terms of phosphate and sulfate. Minor gains were achieved in phosphate resorption in the control treatment, where all three lines showed similar phosphate before and after senescence, while wild-type trees had a significantly higher phosphate content in senescent leaves (Fig. 4.6). Unexpectedly, sulfate resorption efficiencies in the transgenic lines increased from 0 % in the control to 31.1 %, 32.4 % and 36.8 % in lines 1, 2, and 3, respectively. This indicates that barriers to resorption in hybrid poplar may be, in part, the controlled efflux of nutrients from the vacuole through anion channels. Efflux from the vacuole is passive and occurs along the individual anion electrochemical gradients. For phosphate in particular, Pratt et al. (2009) demonstrated that when methylphosphonate (MeP), a non-metabolically active phosphate mimic, was supplied to phosphate-deficient cells it prevented the efflux of phosphate from the vacuole. During senescence, the degradation of the organic-phosphorus pool may maintain high cytosolic phosphate, thus inhibiting the release of vacuolar stores. Increasing the rate of phosphate efflux from the cytosol through the expression of StPht1-1 may have allowed for greater efflux of anions from the vacuole. However, why sulfate would be the preferred anion for resorption with the upregulation of a phosphate transporter is unknown.

The mobility and transport of anions across the tonoplast has historically not been well understood. Recent studies in rice and *Arabidopsis* have begun to characterize the elusive anion channels, such as AtPht5-1 and OsSPX-MFS1, that mediate the influx of anions (with phosphate as the preferred anion) across the tonoplast. Overexpression of these channels led to increased sequestration within plant vacuoles (Liu et al., 2015, Liu et al., 2016). Like the Pht1 family of transporters, they are a part of the major facilitator superfamily (MFS) which is one of the largest families of membrane transporters (Pao et al., 1998). Unlike Pht1 proteins, they contain a well-conserved domain known as SPX, a sequence which has been linked to the identification of key proteins in phosphate homeostasis in all major eukaryotes from *Caenorhabiditis elegans*, to *Drosophila melanogaster*, to *A. thaliana*, to humans (Secco et al., 2012). In plants, studies in *Arabidopsis* and rice have been at the forefront of this research, with a vast array of effects

documented from the mis-regulation of SPX-MFS genes. Localization of these proteins in the cell is also quite variable (Secco et al., 2012). To date, the most promising of the SPX-MFS proteins for improving the phosphate resorption proficiency of plants is OsSPX-MFS3, a constitutively expressed tonoplast-bound low-affinity phosphate transporter that can facilitate phosphate efflux from the vacuole based on external pH and phosphate concentrations. Overexpression of OsSPX-MFS3 in rice resulted in significantly lower vacuolar phosphate in both leaves and roots (Wang et al., 2015). A corresponding gene in *Arabidopsis* has yet to be discovered and phylogenetic analysis of SPX-MFS homologues between the two species suggests that monocot and eudicot genes may have evolved independently, possibly resulting in functional variation of SPX-MFS proteins. The phylogenetic analysis of SPX-MFS grouping with those from tomato (*Solanum lycopersicum* L.) and grapevines (*Vitis vinifera* L.), separate from those found in *Arabidopsis*, suggesting different selective pressures acting on phosphate homeostasis in the two plant species used in this study (Liu et al., 2016).

#### 4.5.4 CONCLUSIONS

Constitutive plant-wide expression of the low-affinity MFS transporter StPht1-1, demonstrated that the barriers and controls of resorption differ in *A. thaliana* and hybrid poplar. In *A. thaliana*, the overexpression of a low-affinity root phosphate transporter caused toxicity symptoms in plants grown in high-phosphorus conditions. No changes to resorption were observed between wild-type and the transgenic lines, suggesting that nutrient status and sink strength have a greater effect on nutrient resorption than the expression of StPht1-1. In hybrid poplar, plant-wide abundant expression of StPht1-1 increased leaf phosphate content in excess phosphorus conditions. Improved phosphate resorption efficiency and to a larger extent sulfate resorption efficiency were apparent in all transgenic lines during senescence. Given that the effects of age, sink strength, and timing of abscission on phosphorus resorption is limited in part by the rate of export of phosphate from the cell. Of greater significance may be the efflux of phosphate reserves out of the vacuole. For the purposes of increasing phosphate remobilization and improving the phosphorus phytoremediation potential of poplar, increased content under excess conditions with no effect on

biomass production is a positive step forward; however, further gains to phosphate resorption efficiencies and proficiencies are required.

### 4.5.5 FUTURE RESEARCH

Creation of double transgenic lines could be warranted to explore the tandem effects of increased transporter presence at the tonoplast and plasma membrane. 35S::StPht1-1 driven expression in conjunction with a poplar cysteine protease promoter driving expression of OsSPX-MFS3 has the potential to allow for normal plant growth and improved remobilization from the main phosphate storage pools. Concurrent exploration into the characterization and mis-regulation of poplar SPX-MFS could have future implications in breeding trees for phytoremediation applications and improved nutrient-use efficiencies. Extrapolation to potential gains in nutrient management of perennial cropping systems, such as tree fruits and vines, would also be an avenue of interest.

## 5. CONCLUSIONS

Global phosphorus scarcity is a fundamental issue that affects our ability to meet future food demands. Large minable phosphate rock deposits, from which we supply agricultural needs world-wide, are a non-renewable resource with depletion expected to occur within the next 30 to 300 years (Cordell and Neset, 2014). Simultaneously, phosphorus has been classified as a pollutant due to increased concentrations in aquatic environments, leading to algal blooms, eutrophication and reduced water quality (Schindler et al., 2008). Agricultural diffuse sources are the primary contributors to phosphorus run-off, and downstream effects are further exacerbated by the high phosphorus storage potential of soil, where sediment can remain rich in phosphorus for decades after nutrient management practices are put into place (Hamilton, 2012). In this thesis, I examined the biological potential for using fast-growing, high phosphate-accumulating poplar and willow planted along field edges and riparian areas to reduce diffuse source introduction of phosphorus in aquatic environments. A caveat to this is that genotypes chosen must also have high resorption efficiencies in order to limit the release of phosphorus during autumnal senescence, and mechanisms for phosphorus resorption in woody perennial species are not well understood (Rennenberg and Herschbach, 2013).

Many species have some level of luxury phosphate uptake, but few have effective phosphorus resorption efficiencies and proficiencies. The majority of research into plant molecular mechanisms of phosphorus resorption has occurred in crop plants with a focus on acquisition and mobilization in nutrient-deficient conditions (Veneklaas et al., 2012). This is not reflective of field conditions, as neither annual nor perennial crop species are grown under severe phosphorus-deficient conditions and have some level of phosphate storage in the vacuole. The implications are that research has focused on breakdown of organic phosphorus as the limiting step to resorption when controls over translocation may be a dominant factor, particularly in perennial species (Chapin and Moilanen, 1991).

The strategic planting of poplar and willow trees, along field margins or adjacent to riparian areas, has the potential to remediate agricultural land and reduce the flow of contaminants downstream, while concurrently functioning as a primary feedstock for a growing fuel and fibre industry. Genotypic screenings combined with genetic manipulation are powerful tools with which to identify or introduce variability in a desirable trait, providing a platform on which to discern the underlying molecular mechanisms. In this thesis, I screened commercially available Salicaceae hybrids for variation in phosphate uptake, storage and resorption. Rapid and cost-effective methods for the large-scale phenotyping of tree metabolites including organic acids, inorganic anions, and soluble sugars using HPLC techniques were established to aid breeding programs with screening and tree selection. This allowed low- and high-phosphate accumulators to be compared and to assess what metabolic trade-offs may exist when accumulating phosphate beyond what is needed to maintain routine plant metabolic processes. The results indicated that resorption was the largest factor impeding the efficient phytoremediation of phosphorus-rich sites and so barriers to resorption in poplar were investigated through transgene expression in poplar. This thesis thereby addresses both practical and theoretical aspects of nutrient resorption. From a practical aspect, the findings of this thesis allow for commercial varieties to be recommended for field trials. Future deployment of promising varieties to producers for use in the field would aid in limiting diffuse phosphorus entry into riparian ecosystems. An effective method with which to rapidly assess infield performance was also established. From the theoretical aspect, barriers and mechanisms of phosphate resorption were investigated. With further research, this knowledge of limits to resorption could facilitate the effective comparison of poplar and willow transcriptomes leading to the targeting of nutrient resorption genes in breeding programs for the use of trees in phytoremediation, agriculture and horticulture.

### 5.1 **PHOSPHORUS STORAGE AND RESORPTION IN TREE SPECIES**

Phosphorus accumulation in excessive or deficient nutrient conditions has largely been studied in crop species, with leaves being the primary organ for assessment of plant demand for nutrients (Bollons and Barraclough, 1999; Marschner, 2012). Established optimal leaf concentrations are  $3 - 5 \text{ mg P g}^{-1}$ , with accumulation above 10 mg P g<sup>-1</sup> increasing the probability of detrimental effects on plant growth (Marschner, 2012). Resorption efficiencies of phosphorus for deciduous forests have been reported to be ~ 50 % (Vergutz et al., 2012) with 23 – 60 % observed in hybrid-poplar plantations (Brown and Driessche, 2005). The controlled experiments in this study suggest that Salicaceous tree species have an optimal leaf phosphorus concentration range similar to those found in crop species. Trees capable of luxury uptake, however, have leaf concentrations > 5 mg P g<sup>-1</sup> when given optimal levels of external phosphorus. No toxicity

symptoms were observed even when internal phosphorus concentrations increased beyond 10 mg P g<sup>-1</sup>. For rapid more cost-effective analysis, it was demonstrated that in phosphorus-replete conditions, HPLC measures of phosphate of a single mature leaf can effectively estimate the average total phosphorus from all leaves. Further analysis demonstrated that the majority of phosphorus stored in leaves was present as phosphate in both mature and senescent leaves, suggesting that breakdown of organic phosphorus is not limiting nutrient resorption in poplar. For phytoremediation purposes, the poplar hybrid Tristis has the highest accumulation potential, yet hybrid willow AAFC-5 had the greatest resorption potential, thereby reducing the downstream flow of phosphorus. In summary, poplar did not exhibit high phosphorus resorption efficiencies or proficiencies while willow had a reasonable resorption efficiency during senescence. These underlying differences in related species allow for improved identification of genes involved in nutrient translocation during future transcriptomic analysis.

# 5.2 DISCERNING THE EFFECTS OF PHOSPHATE STATUS ON THE METABOLISM OF HYBRID POPLAR

Toxicity induced by the overaccumulation of phosphorus in plant cells has been documented in poplar (Teng and Timmer, 1990), and metabolic adjustments for accumulated anions must be made for the cells to maintain pH and remain electrically neutral (Roberts, 2006). Toxicity symptoms or a reduction in growth was therefore expected to occur during growth trials; however, none were observed (Chapter 2). Subsequent metabolite analysis suggested that the increasing potassium concentrations had a larger effect on poplar plant cells than the excess phosphate accumulation; therefore, there appears to be no significant metabolic trade-offs for the luxury uptake of phosphate.

The progression of phosphate resorption during senescence (as indicated by chlorophyll degradation) was also examined. It was apparent that phosphorus accumulation continued well past the collection date of mature leaves at approximately bud-set. In senescent leaves, poplar and willow both had resorption proficiency values above > 0.08 % indicating incomplete resorption, as defined by Killingbeck (1996). Given that nutrient resorption proficiency is a measure of the selective pressures against nutrient loss, it is a trait that could be selected for as part of a breeding program. When phenotyping, experiments done in Chapter 3 show that leaf phosphate concentrations at 60 % of initial chlorophyll levels can be used to select genotypes with higher

resorption proficiencies. This coincides with ecological observations that phosphate remobilization occurs during the first two stages of senescence, bud-set and the reduction in chlorophyll content (Keskitalo et al., 2005). Once again, indications are that translocation of phosphate from the leaf is limiting resorption from the leaf. A comparative expression analysis of Pht1 family transporters in leaves with 60 % chlorophyll for the poplar species of P39 (no resorption) and Tristis (32 % resorption efficiency) implicate PtPht1-9 and PtPht1-12 in senescence. The mis-regulation of these genes in poplar could lead to a better understanding of the barriers to nutrient resorption in perennial species.

## 5.3 CONSTITUTIVE EXPRESSION OF AN EXOGENOUS PHT1 FAMILY MEMBER IN ANNUAL AND PERENNIAL SYSTEMS.

Previous experiments in Chapter 2 and 3 implicate translocation across the plasma membrane (either out of the cell or into the phloem), as the primary control on phosphorus resorption in perennial species under phosphorus replete conditions. This has previously been suggested in the literature as typical determinants such as age, source-sink interactions and timing of abscission have a little to no correlation to phosphorus resorption (Killingbeck et al., 1990; Chapin and Moilanen, 1991). High internal loads of phosphate in leaves both before and after autumnal senescence in poplar and insufficient resorption in willow genotypes (Chapter 2 and 3) indicate that improvements can be made. Since the primary barrier to translocation is thought to be the plasma membrane, a well-characterized, low-affinity member of the Pht1 H<sup>+</sup>/H<sub>2</sub>PO<sub>4</sub><sup>-</sup> symporters family (StPht1-1) was chosen for constitutive expression in hybrid poplar. A. thaliana was also transformed to observe if similar barriers to resorption exist in an annual species. Constitutive expression of potato phosphate transporter StPht1-1 in A. thaliana led to impeded growth under high external phosphate conditions and no effect on phosphate resorption. This may be because of the greater role that sink-strength, such as seed production, is thought to play in nutrient resorption in short-lived species (Güsewell, 2005). In the hybrid poplar P39, StPht1-1 expression led to small gains in phosphate resorption and large improvements in sulfate resorption from the leaves. Resorption is therefore limited, in part, by the rate of phosphate export from the cell, but the release of anion reserves from the vacuole may be of greater significance. Phosphate is released from the vacuole in deficient conditions to sustain metabolic processes occurring in the cytoplasm. Conversely, sufficient cytosolic phosphate inhibits phosphate efflux from the vacuole

(Pratt et al., 2009). I theorize that increasing the transport of phosphate out of the cytosol via StPht1-1 expression led to the opening of anion channels on the tonoplast. While such channels can have a preference for phosphate, other anions such as sulfate are able to cross and travel along their concentration gradient (Liu et al., 2015). Since poplar species had greater sulfate resorption than willow in the greenhouse trials (Chapter 3), poplar may have pre-existing mechanisms for sulfate resorption resulting in rapid translocation to sink tissues. As poplar species had smaller gains in phosphorus resorption, it is thought that StPht1-1 was not able to establish a strong enough concentration gradient to efflux large amounts of phosphate from the vacuole. It may be that the pH shift with release of anions from the vacuole was rapidly balanced by proton pumps limiting  $H^+/H_2PO_4^-$  symport out of the cytosol. Conversely, sulfate may have multiple ways to transverse the plasma membrane. There are still many questions surrounding phosphate translocation and this research provides a platform from which to study resorption during senescence in perennial tree species.

### 5.4 LIMITATIONS OF RESEARCH

One of the primary limitations of this study was that the growth conditions used do not accurately reflect field conditions. Trees were grown from cuttings in a greenhouse setting with nutrients provided in optimal ratios for growth. Field trials would show much higher variability due to different soil types, anion exchange capacities, nutrient profiles, as well as other environmental stressors, such as incidence of drought and disease. Furthermore, senescence occurred in outdoor temperatures in Vancouver B.C., which rarely fall below 0 °C in the winter months, compared to where these commercial genotypes are grown on the Canadian Prairies and exposed to average winter low temperatures around -20 °C. Senescing in a milder climate would prolong leaf residence times, increasing the time in which nutrients could be resorbed. Over the course of the experiment, trees were grown for a maximum of seven months rather than the three to four field seasons that is done in short-rotation coppicing. Given that each tree was grown under greenhouse conditions and was young at the time of harvest, values mentioned in this study should be considered as optimal or relative values rather than indicative of genotypic performance in field settings.

A second limitation is that constitutive expression of an exogenous gene is not an optimal method for proof-of-function experiments. Optimally, RNAi knock-down lines of genes of interest

would concurrently be used to show a causal effect on nutrient resorption during senescence. However, due to lack of information on the molecular mechanisms of phosphate translocation, transporter functional redundancy, the role of transporters in normal plant growth and the duplicative nature of most gene families (i.e. redundancy) in poplar, I was unable to select a single suitable target gene for knock-down. Future prospects of multiple gene knock-out mutants using CRISPR/Cas9 in poplar are promising for further investigation into the molecular barriers of nutrient resorption.

### 5.5 APPLICATIONS AND FUTURE RESEARCH

The conclusions of this thesis have multiple implications on the establishment and focus of future tree breeding programs targeting phytoremediation. Relative performance and capabilities of current commercial poplar and willow trees in the removal of excess phosphorus from phosphorus-rich soil were estimated (Chapter 2), allowing for rapid deployment in the field and future assessments of their effects on reducing diffuse-source phosphorus pollution. The verification of the functionality of a high-throughput HPLC method for phosphate determination from a single leaf was done and will provide a fast, effective way for assessing performance of these trees in the field. This high-throughput method in conjunction with identifying a level of chlorophyll senescence at which to measure resorption proficiency in poplar and willow (Chapter 3) will allow for breeding programs to select trees with improved nutrient resorption capabilities. Finally, building off of the transgenic StPht1-1 lines (Chapter 4) and performing a comparison of the poplar and willow transcriptomes in post-senescence tissues and under high-phosphate conditions from Chapter 1 may identify genes most likely to be involved in phosphate translocation and establish a platform for future research in resorption. This would have impacts on future tree breeding for phytoremediation and could be extrapolated to perennial crop species such as grapes, apples and blueberries for improved crop nutrient management. As steps towards this end goal, future research prospects have been outlined below.

## 5.5.1 COMPARISON OF POPLAR AND WILLOW TRANSCRIPTOMES FOR IDENTIFICATION OF PHOSPHATE TRANSLOCATION GENES

In the experiments outlined in Chapter 2, several genotypes had promising and contrasting phenotypes that could be used to identify genes that affect phosphate translocation during senescence, as well as assess their role during normal plant growth. Genotypes to be analyzed would be poplar hybrids Tristis (high luxury consumption, some resorption) and P39 (low luxury consumption, no resorption), and willow hybrid AAFC-5 (no luxury consumption, highest resorption). Questions to be addressed with these data would be as follows:

- Can gene expression explain the difference in phosphate uptake?
- What genes are affected by high internal phosphate concentrations?
- What genes allow for better phosphate proficiency?

The first question would be addressed by comparing pre-senescent leaf and root tissue of Tristis, P39 and AAFC-5 grown at optimal nutrient conditions (100N:13P). The second would look at differences in gene expression in Tristis pre-senescent leaf tissue grown in optimal (100N:13P) and excess (100N:70P) nutrient conditions. For the final question, transcriptome data would be compared from Tristis, P39 and AAFC-5 leaf tissue undergoing senescence with 60 % of chlorophyll remaining. This analysis could also be used to explore ties between phosphate and sulfate resorption, as poplar was observed to have higher sulfate resorption than willow.

#### 5.5.2 MIS-REGULATION OF POPLAR PHT1 FAMILY TRANSPORTERS.

In Chapter 3, from semi-quantitative expression data, I hypothesized that poplar Pht1 family transporters PtPht1-9 and PtPht1-12 play a role in leaf remobilization and resorption of phosphate during senescence. RNAi knock-down of these genes in poplar could help elucidate their roles in phosphate transport, although care would have to be taken to prevent silencing other transporters with high sequence similarity (Loth-Pereda et al., 2011). Simultaneously, the similarity could be used to some advantage to reduce the expression of multiple gene family members with a single construct.

#### 5.5.3 PHT1 TRANSPORTERS IN ARABIDOPSIS

Although no phenotypes were found for the *A. thaliana* T-DNA line used in this study (Chapter 4), phenotypes have been reported in the literature for various phosphate transporter mutants (Shin et al., 2004; Nagarajan et al., 2011; Ayadi et al., 2015). Seeds from all transporters implicated in translocation, the *pht1-1*, *pht1-4*, *pht1-5* mutants and the *pht1-1/pht1-4* double mutant, could be obtained from the studies listed above and screened for differences in phosphate resorption from the leaves and phytic acid content of the seeds. The complementation of these mutant backgrounds could then be done by inserting functional copies of the genes, driven by the

promoter of a cytokinin-signaling gene, as reduction in cytokinin is the signal that initiates senescence (Breeze et al., 2011). By linking the complementation temporally to non-senescence related genes, function during senescence could be demonstrated without having to interpret the results through a potentially confounding growth phenotype. These would be the first steps to elucidating molecular resorption controls in annual species.

#### 5.5.4 VACUOLAR EFFLUX AS A BARRIER TO PHOSPHATE RESORPTION

Constitutive expression of StPht1-1 in poplar yielded slight improvements to phosphate resorption efficiency (Chapter 4), leading to the hypothesis that lack of efflux from the vacuole may be preventing further gains. OsSPX-MFS3 in rice has been characterized as a low-affinity phosphate transporter located on the tonoplast that mediates phosphate efflux from the vacuole (Wang et al., 2015). Should OsSPX-MFS3 be driven by a senescence-associated gene promoter and transformed into wild-type P39 and P39 35S::StPht1-1 (line 3) plants, this hypothesis could be verified. To my knowledge, no senescence-associated promoters in poplar have been characterized to date. A potential candidate would be the cysteine protease gene Potri.009G098100, which has been used as an indicator of senescence induction in several studies (Loth-Pereda et al., 2011). The sequence length required for a functional Potri.009G098100 promoter will need to be determined before cloning and subsequent use in a construct with OsSPX-MFS3. Characterization of a senescence-associated promoter in poplar has the potential to contribute to a wide range of studies focusing on the molecular mechanisms of senescence in perennials. The expression of a vacuolar efflux transporter will determine if further resorption controls lie farther downstream and involve phosphate loading in the phloem. The molecular mechanisms regulating nutrient translocation and resorption have yet to be explored in perennial species and have the potential to impact tree breeding programs for phytoremediation, as well as improve nutrient management in perennial crop species.

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## **APPENDIX A – CLONING VECTORS**

Vector maps for Gateway® pENTR entry vector and Gateway® destination vectors pH7WGY2, pH7YWG2 and pK2GW7. The pENTR map was retrieved from lifetechnologies.com. Destination vector maps were retrieved from gateway.psb.ugent.be.



(c) = complementary strand



DESTINATION VECTOR USED FOR Arabidopsis transformation with N-terminus YFP tag.



DESTINATION VECTOR USED FOR Arabidopsis transformation with C-terminus YFP tag.



#### Destination vector used in P39 transformation