DISSERTATION

A NEW WOODY PERSPECTIVE ON COPPER HOMEOSTASIS: SYSTEMIC COPPER TRANSPORT AND DISTRIBUTION, EFFECT OF COPPER ON LIGNIFICATION, AND WATER TRANSPORT IN HYBRID POPLAR

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ABSTRACT

A NEW WOODY PERSPECTIVE ON COPPER HOMEOSTASIS: SYSTEMIC COPPER TRANSPORT AND DISTRIBUTION, EFFECT OF COPPER ON LIGNIFICATION, AND WATER TRANSPORT IN HYBRID POPLAR

Copper (Cu) is an essential micronutrient for plants. Chapter 1, as background for this dissertation, reviews the functions and homeostasis of Cu. We know at the cellular level how Cu is delivered to target proteins in the chloroplasts, thus explaining in a large part why Cu deficient plants have reduced photosynthetic capacity. However, Cu is also a cofactor of lignin polymerization enzymes that affect cell wall and xylem structures required for water and mineral transport. How Cu deficiency affects water transport, mineral nutrition, and photosynthesis at a whole plant level is underexplored. To address this knowledge gap, we used hybrid white poplar as a model. In chapter 2, a stable isotope method to trace Cu movement in poplar tissues was coupled with analysis of photosynthesis and stomatal conductance. Upon resupply of Cu, priority targets identified were stems and younger leaves which recovered quickly and was associated with higher stomatal conductance. In chapter 3, the effect of Cu deficiency on the elemental composition of leaves and stems of different age were analyzed. Interestingly, tissue type and age, as well as Cu deficiency, were found to all significantly affect within-plant nutrient partitioning patterns. In chapter 4, the effects of Cu deficiency on cell wall chemical composition and water transport traits were determined. Although Cu deficiency strongly affected cell wall chemistry, it did not significantly impact hydraulic capacity nor the density and size of xylem vessels in stems. However, Cu deficiency resulted in markedly stiffer mesophyll cell walls, possibly arising from changes to cell wall chemistry or structure. Together, these results, as discussed in chapter 5, indicate that although xylem lignification was adversely affected by Cu

deficiency, the water transporting vessels remained largely unaffected, thus allowing efficient recovery. This work opens new avenues to explore the effects of plant nutrition on whole-plant physiology and function.

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CHAPTER 1: A WHOLE-PLANT PERSPECTIVE ON COPPER HOMEOSTASIS

1.1 INTRODUCTION

Plants require nutrients from the soil to complete their life cycle. In turn, people obtain a portion of their nutrient requirements by consuming plant parts (Printz *et al.,* 2016). Nutrients are needed in varying amounts and are classified as macronutrients (needed at 1000 mg/kg DW-1 or higher) or micronutrients (needed at 0.1-100 ppm) (Marschner 2012, Printz *et al.,* 2016, Epstein *et al.,* 1999). Essential macronutrients are nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S). Essential micronutrients include copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn) (Marschner 2012). One mineral micronutrient needed for proper plant growth and physiology is copper (Cu) metal. Cu homeostasis has been studied extensively at the cellular level and much is known about the proteins that use Cu as cofactor to accomplish key biochemical redox reactions.

1.2 THE ROLE OF CU IN PLANT BIOLOGY

Cu proteins

In plants, Cu is an essential micronutrient that plays a cofactor role in several enzymes in photosynthesis, mitochondrial respiration, cell wall metabolism, and reactive oxygen species (ROS) metabolism (Pilon *et al.,* 2006). In chloroplasts, which function in photosynthesis, Cu is present as a cofactor in the protein plastocyanin (PC). PC functions as an electron carrier to drive photosynthesis by carrying an electron from the cytochrome b_6f complex to photosystem I (Weigel *et al.,* 2003). Furthermore, Höhner *et al* (2020) recently provided evidence that PC is the only long distance electron carrier between cyt-b_6f and PSI and not plastoquinone (PQ). This was characterized in the *curt1abcd Arabidopsis* loss-of-function mutant that had higher grana diameter (~1600nm), thus varying the distance the two electron carriers must travel (Höhner *et al.,* 2020). By measuring the time, it takes PC to travel from cyt-b_6f to PSI (220-300 µs) in the WT and greater than 6 ms in the *curt1abcd Arabidopsis* mutant, these data suggest that it takes

PC less time to transfer electrons to PSI versus PQ (Höhner *et al.,* 2020). Cu also shares a role with Zn in Cu/Zn superoxide dismutase to remove superoxide and form hydrogen peroxide (Pilon *et al.,* 2011). Another functional role of Cu is the use in the laccase enzymes for lignin polymerization in the plant cell wall (Lu *et al.,* 2013). The redox state of Cu is typically Cu^{+} or Cu^{2+} while operating as a protein cofactor.

Cu uptake, transport, and distribution across the whole plant

Cu is present in green tissues of plants at a concentration range of 2-50 µg/g DW (ppm) (Epstein *et al.,* 2005, Burkhead *et al.,* 2009). These levels are species specific and can be different depending on the bioavailability of Cu in the growth substrate (Burkhead *et al.,* 2009). While present in the soil at concentrations of 55 ppm on average, it must be taken up into plants via the root system (Burkhead *et al.,* 2009). One strategy used in investigating Cu uptake systems is to deplete plants of Cu and use molecular genetics to find upregulated proteins during and after deficiency. It has been suggested that ferric reductase oxidases 4/5 (FRO4/5) act in reducing Cu^{2+} to Cu^{+} preparing it for uptake by COPT1, which is a COPT/Ctr-like protein family member in roots (Bernal *et al.,* 2012). Cu can perhaps also be taken up by ZIP2 and ZIP4 as Cu^{2+}, which are expressed under Cu deficiency (White *et al.,* 2009). ZIP2 and ZIP4 are not specific Cu importers but have been suggested to transport other divalent cations as well (Wintz *et al.,* 2003; Del Pozo *et al.,* 2010). COPT2 is a possible alternative transporter that can mediate Cu uptake into plant roots and is expressed in shoots (Perea-García *et al.,* 2013). Once Cu has made it into the root cells, it may be stored in the vacuole and then exported back to the cytosol when needed via the tonoplast localized COPT5 protein, as suggested by studies in *Arabidopsis* (Klaumann *et al.,* 2011).

While Cu may be needed in cellular processes in the roots, it also must be shipped to above ground tissue for utilization in the leaves and stem. This involves loading Cu into the xylem sap for long distance transport to these organs. In rice, OsHMA5 (Heavy Metal Associated) is proposed to transport Cu as Cu^{+} and knockouts in HMA5 were shown to

accumulate Cu in roots (Deng *et al.,* 2013). In *Arabidopsis*, HMA5 is expressed in roots and its expression is induced by Cu (Andres-Colas *et al.,* 2006). There are several bodies of evidence that support Cu chelation to various stable complexes (e.g., proteins, small molecule chelators) to achieve long distance transport in Arabidopsis and rice (Printz *et al.,* 2016). The challenging factor is that most of the chelators involved in Cu transport can also facilitate transport and binding of other metals. The non-proteinogenic amino acid nicotianamine (NA) is believed to chelate Cu for transport in xylem sap. However, NA can bind other transition metals (Curie *et al.,* 2009). Ryan *et al.,* (2013) suggested that Cu gets re-oxidized for long distance transport in tomato based on Cu speciation analysis with X-ray absorption spectroscopy (XAS) but, these studies were done under Fe deficient conditions. Metallothioneins (MTs) are proteins that bind Cu+ and are expressed in phloem and mesophyll cells of Arabidopsis (Guo *et al.,* 2003). Arabidopsis MT knockouts had lower Cu in the seeds versus a wild type. This observation suggests MTs function in mobilizing Cu for transport to young developing tissues most likely via the phloem (Benatti *et al.,* 2014). Zheng *et al.,* (2012) discovered OsYSL16 (Yellow Stripe Like) in rice to be a Cu-chelate transporter localized in the phloem. It was proposed that OsYSL16 functions in loading Cu into the phloem for transfer up to developing leaves and seeds (Zheng *et al.,* 2012). Chen *et al.,* (2011) showed that YSL3 plays a role in Cu accumulation in 2 Arabidopsis shoots. Another protein involved in Cu movement in above ground tissues is COPT6. COPT6 is located in the plasma membrane and mediates Cu distribution to leaves and seeds under Cu limitation (Jung *et al.,* 2012). Generally, it is accepted that Cu is transported in stem as Cu^{2+} and is reduced to Cu^{+} by FRO4 for transport into the leaf cells (Bernal *et al.,* 2012; Ryan *et al.,* 2013).

At the leaf level, the chloroplast, the mitochondria, and the cell wall are the main organelles/structures competing for Cu (Shahbaz *et al.,* 2015; Printz *et al.,* 2016). In the leaf, Cu first must enter the mesophyll cell cytosol before it is delivered to several proteins (Aguirre *et al.,* 2016). One of it's stops once inside the cytosol of the mesophyll cell is to PAA1(P-type ATPase

of Arabidopsis) in the chloroplast envelope. Cu can be delivered to PAA1 by the copper chaperone PCH1 via direct interaction (Blaby-Haas *et al.,* 2014). This ultimately delivers Cu ions into the stroma where it gets transferred to Cu/Zn SOD by CCS (the copper chaperone for superoxide dismutase) and serves to remove superoxide radicals (Abdel-Ghany *et al.,* 2005). Cu is also required in the thylakoid lumen, where it becomes a cofactor in plastocyanin (PC) and gets there by PAA2 import, however it remains unclear how PAA2 receives the Cu ions. (Tapken *et al.,* 2012). Cu is also delivered to laccase enzymes, which require 4 Cu ions per molecule of enzyme synthesized (Berthet *et al.,* 2011).

Cu deficiency, toxicity, and regulation

Reported Cu deficiency symptoms in plants are stunted growth, chlorosis and/or necrosis starting at the apical meristem, and wilting of young leaves (Marschner 2012). Despite being an essential micronutrient Cu can be toxic at the cellular level by generating hydroxyl radicals (Rodrigo-Moreno *et al.,* 2013). When Cu becomes limiting in a plant, its homeostasis undergoes a molecular remodeling via the transcription factor SPL7 which becomes active on low Cu (Bernal *et al.,* 2012). SPL7 up-regulates the expression of COPT1, COPT 2, and COPT 6 and also FRO4/5 (Yamasaki *et al.,* 2009). SPL7 also turns on the expression of several microRNAs that serve to mediate the degradation of mRNAs encoding for target Cu proteins (Yamasaki *et al.,* 2009). Some examples include: Cu/Zn SOD, polyphenol oxidase (PPO), laccase family members, and CCS (Pilon 2017). PC is not regulated by microRNA expression, and it is thought that “non-essential” Cu proteins are downregulated so that the available Cu pool can be shipped to developing tissues for insertion in PC. This is known as the Cu economy model and has been supported by evidence in *Arabidopsis* and poplar (Ravet *et al.,* 2011; Shahbaz *et al.,* 2015; Abdel-Ghany *et al.,* 2008).

Stable isotopes of Cu in plants

Cu has two stable isotopes: ^{63}Cu and ^{65}Cu present at 69.15% and 30.85% in the earth’s crust, respectively (Savage 2018). Stable isotopes are commonly used in plant research to examine

the movement of minerals in plants to obtain information on metabolic fate, utilization, and distribution. As mentioned previously, Ryan and coworkers (2013) used the stable isotopes of Cu to investigate Cu translocation mechanisms in tomato and oat under Fe deficiency. Cao *et al*. (2020) used stable Cu isotopes to show Cu mobility in the xylem and the phloem in willow based on Cu in the xylem and phloem sap (*Salix integra*). Similarly, Zheng and colleagues (2012) fed ^{65}Cu-NA to rice plants at an excised leaf to track its redistribution in wild type and *OsYSL16* knockouts. Results from the isotope tracer experiments revealed that the ^{65}Cu-NA was the complex that moved into younger tissue versus the $^{65}CuCl_2$ control (Zheng *et al.,* 2012). These data illustrate the efficacy and physiological information that can be gained from the use of stable isotopes in plant nutrition. However, there is little information about Cu translocation to photosynthesis using stable isotopes.

1.3 POPLAR TREE USE, GROWTH, AND DEVELOPMENT

Poplar use as a crop and model system

Tree species require proper nutrition and provide the world with fiber, biofuels, and building materials (e.g., wood). Some of the products in North America made from poplar wood include paper, plywood, veneer, and chopsticks (Balatinecz *et al.,* 2001). According to the Food and Agricultural Organization, global production and trade of forest products was at its highest in 2018 (FAOSTAT). Furthermore, *Populus* has grown increasingly important in the forest product industry but also serves as a model tree in plant biology research (Bradshaw *et al.,* 2000). A full sequence of the poplar genome (*P. trichocarpa*) has been available since 2004 (Ma *et al.,* 2004). A draft genome of hybrid white poplar (*P. tremula x P. alba*) is also available (Mader *et al.,* 2016). Poplar is useful as a model tree due to its fast growth, ease of vegetative propagation, and transformation with *Agrobacterium tumefaciens*. It also provides a new model tree organism to integrate physiological and molecular experiments (Bradshaw *et al.,* 2000; Ravet *et al.,* 2011; Shahbaz *et al.,* 2015).

Cu and lignification

There is evidence dating back to the 1960's that lignification of plant cell walls can be affected by Cu supply (Marschner 2012, Oldenkamp *et al.,* 1966). Oldenkamp *et al.* (1966), observed deformed stems of Douglas fir trees that were confirmed to be Cu deficient in their needles. Under Cu deficiency in wheat leaves, lignin content was reduced by almost half with an increase in alpha-cellulose content (Marschner 2012; Robson 1981). It has also been suggested that Cu deficiency results in reduced lignification of sunflower stems (Bussler 1981). Increased Cu supply has been shown to increase lignin content in soybean roots (Lin *et al.,* 2005). The lignin content increased in soybean roots within 24 to 72 hours after treatment with 10 µM Cu (Lin *et al.,* 2005). In *Brassica juncea*, it was shown that exposure to Cu oxide nanoparticles (CuONP) caused an increase in lignification in hypocotyls based on phloroglucinol-HCl staining with a particular increase in xylem vessel lignification (Nair *et al.,* 2015). The phloroglucinol-HCl staining also revealed increased lignification in the roots with increased exposure to CuONP (Nair *et al.,* 2015).

The role of Cu in lignification is through the laccase enzymes (Lu *et al.,* 2013). Laccases are Cu containing glycoproteins and function in oxidizing monolignols from the phenylpropanoid pathway in the apoplast (Lin *et al.,* 2005; Printz *et al.,* 2016). Zhao *et al.* (2013) generated a triple mutant *lac4lac11lac17* in *Arabidopsis* that had an extreme dwarf phenotype, drastically reduced lignification, and reduced *LAC* expression. Interestingly, the triple mutant did produce a stem, but it had a different vascular arrangement compared to the wild type (Zhao *et al.,* 2013). One laccase enzyme isolated from the stem of loblolly pine was shown to complete *in vitro* oxidation of monolignols (Bao *et al.,* 1993). Interestingly, Lu *et al.* (2013) presented results that suggest Ptr-miR397a is negative regulator of laccase genes in *P. trichocarpa*. In this study, overexpression of this miRNA was shown to reduce lignin content (Lu *et al.,* 2013). Berthet *et al.* (2011) presents evidence that *LAC4* and *LAC17* disruption causes alteration to lignification in *Arabidopsis* stems. In a recent study, copper-containing uclacyanin (UCC) proteins UCC1 and

UCC2 were shown to be required for lignification in a central nanodomain in the Casparian strip of *Arabidopsis* (Reyt *et al.,* 2020). Specifically, UCC1 was shown to localize to the central domain of the Casparian strip compared to other Casparian strip-located proteins (Reyt *et al.,* 2020). These data together suggest that Cu is involved in the lignification process in plants through laccase oxidation of monolignols and in some cases are required for lignification (Reyt *et al.,* 2020).

Xylem anatomy and physiology of Populus

Poplar trees are woody, dicotyledonous plants where xylem (wood) undergoes primary and secondary growth (Myburg *et al.,* 2013). The wood is classified as diffuse porous meaning all the vessels are of similar diameter throughout the growth ring (Blake *et al.,* 1996) The chemical composition of poplar wood consists of 50% cellulose, 30% hemicellulose, and 20% lignin (Balatinecz *et al.,* 2001). Poplar xylem is made of primarily vessel elements, fiber cells, and parenchyma cells (Myburg *et al.,* 2013). Vessel elements follow this sequence of cell growth and differentiation: cell division and enlargement, cell wall thickening, lignification, and programmed cell death. As such, xylem vessel elements are dead at maturity (Myburg *et al.,* 2013). As the protoplast grows and differentiates it enlarges its plasma membrane, primary wall, and eventually a secondary wall. The secondary wall undergoes a thickening process where cellulose, lignin, hemicellulose, and proteins are laid down and polymerized (Myburg *et al.,* 2013; Printz *et al.,* 2016). Vessel elements are the primary conduits for water transport in dicotyledon plants and must be able to withstand the negative pressures that arise within the transpiration stream (Myburg *et al.,* 2013).

1.4 CONCLUSIONS AND OPEN QUESTIONS IN THE FIELD

Biologically important Cu-containing and Cu transport proteins have been studied extensively. The copper deficiency response has also been characterized in *Arabidopsis* and poplar. In contrast, the mechanism by which Cu is delivered to the leaf from the roots for photosynthetic

and other metabolic uses is still largely unknown. Whole-plant and organ level studies in *Arabidopsis* have indicated that mineral nutrient content and concentration can fluctuate over time in various organs. Therefore, our understanding of Cu transport and distribution at the level of the chloroplast, leaf, and whole-plant constitutes a partially painted picture. A better understanding of the mechanisms that mediate systemic mineral utilization can help us to devise strategies for improving plant productivity. We have good insight at the cellular level in the leaf and root surface but a very poor understanding of how Cu is allocated systemically. How is it distributed/partitioned across the whole plant? What are the processes that affect its long-distance transport in poplar? Does leaf age drive the prioritization of Cu upon resupply?

Furthermore, the structures that support water transport (e.g., xylem vessels) and nutrient delivery to target tissues depend on lignification of their secondary cell walls, thus the presence of Cu in laccase enzymes. Much effort in lignin research has shown that knocking out several *LAC* genes causes morphological changes and reduced lignification in plant vasculature. Interestingly, *LAC* gene expression of several transcripts returns upon Cu resupply after 5 weeks of deficiency in hybrid poplar. Additionally, laccases are confirmed to be nonredundant with Fe-containing peroxidases for lignin polymerization in *Arabidopsis.* Laccases have also been shown to co-localize in xylem secondary cell walls in young, intermediate, and mature stems of *Arabidopsis* tagged with red fluorescent protein. In low-lignin transgenic poplar, reduced water transport efficiency was observed in field grown plants although not due to xylem vessel collapse. These observations lead to several questions regarding Cu homeostasis lignification, and water transport. How does Cu homeostasis play a role in water transport? Is it necessary for optimal vessel structure and sufficient water transport efficiency? How does Cu deficiency alter the morphology and chemistry of poplar xylem, if at all?

1.5 SCOPE OF THIS DISSERTATION

Previously, studies of Cu homeostasis have focused on what is occurring at the molecular level under Cu deficient conditions both in hydroponic culture and agar. It has also focused on how the expression of target transcripts of the Cu-miRNAs change upon Cu resupply. There is also a lack of understanding of the quantitative anatomical changes that occur in the xylem vessels under Cu deficiency. The research in this dissertation focuses on a higher level of organization including the organ and whole plant level.

When Cu was resupplied to Cu deficient hybrid poplar, a three-fold recovery was observed: Cu content in young leaves, photosynthetic electron transport, and plastocyanin expression. This opens the questions of how Cu is moved in plants and what is its physiological priority? As mentioned previously, recent evidence has been published showing that PC is the only long-distance electron carrier in plant chloroplasts (Höhner *et al.,* 2020). Could it be that plants that are deficient in Cu orchestrate a mechanism of transport that shuttles Cu over long-distances to use in PC once resupplied? Figure 1 illustrates the conceptual overview of this dissertation.

I chose a broad and integrative approach to address the questions presented in section 1.4 of this chapter. This approach includes hydroponics, stable isotope chemistry, chlorophyll fluorescence methods, water transport methods, and light microscopy. Chapters 2-4 address the questions from section 1.4 experimentally and Chapter 5 gives a summarizing conclusion to my dissertation research.

1.6 FIGURES AND TABLES

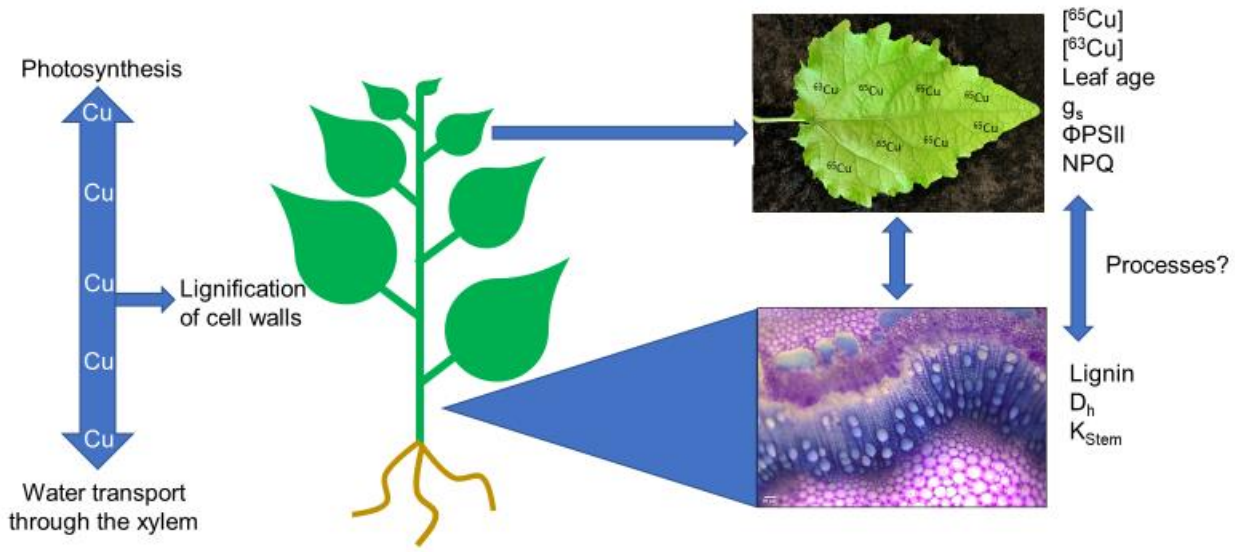

Figure 1. Schematic overview of the processes addressed in this dissertation. Photosynthesis and lignification in the context of Cu homeostasis are the two processes of focus.

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CHAPTER 2: RECOVERY AFTER DEFICIENCY: SYSTEMIC COPPER PRIORITIZATION AND PARTITIONING IN THE LEAVES AND STEMS OF HYBRID POPLAR[1]

2.1 SUMMARY

Copper (Cu) is important for many aspects of plant function including photosynthesis. It has been suggested that photosynthesis, especially in young leaves is prioritized for Cu delivery after deficiency in hybrid poplar (Shahbaz *et al.,* 2015). To determine relative Cu delivery prioritization, we enriched hydroponic plant growth media of Cu deficient poplar with 98% ^{65}Cu and tracked Cu delivery after deficiency to young leaves, mature leaves, and stems. Young leaves acquired ~58% more ^{65}Cu on day 1 and ~65% more ^{65}Cu by day 3 compared to mature leaves. Additionally, stomatal conductance (g_s) was measured on leaves for 6 weeks and during a 3-day ^{65}Cu pulse resupply period. During deficiency, mature leaves maintained a higher g_s than younger leaves but three days after Cu resupply the younger leaves that had recovered showed the highest g_s. In conclusion, these results provide a quantitative understanding of how Cu is systemically transported and distributed to photosynthetic and stem tissues.

2.2 INTRODUCTION

Cu serves as a cofactor in several proteins that are required in important processes e.g., photosynthesis, lignin polymerization, and respiration (Burkhead *et al.,* 2009). Deficiency in Cu therefore critically affects plant function (Abdel-Ghany and Pilon 2008, Marschner 2012). Reported Cu deficiency symptoms in herbaceous plants include stunted growth, chlorosis and/or necrosis starting at the apical meristem and wilting of young leaves (Epstein and Bloom 2005, Marschner 2012). Cu deficiency can also stunt tree growth as has been observed in *Pinus radiata* (Ruiter 1969). Cu delivery at the organ level, its distribution across the whole

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plant, and the traits that affect its long-distance transport are part of a physiological process in trees with unanswered questions. Shahbaz and co-workers (2015) examined the response over 5 days to Cu resupply in Cu deficient hybrid poplar. These researchers reported a prioritization of Cu delivery to younger leaves and the subsequent recovery of plastocyanin abundance, Cu content, and chlorophyll fluorescence parameters (e.g., photosynthesis) within a period of 3 days after the start of Cu resupply. These results suggested that photosynthesis in younger leaves could be the physiological priority for Cu delivery (Shahbaz *et al.,* 2015).

It is likely that physiological and environmental cues (e.g., stomatal conductance and light) are integrated with Cu homeostasis in plants (Zhang *et al.,* 2014, Yan *et al.,* 2017, Rahmati *et al.,* 2020). It has for instance been demonstrated that stomatal conductance correlates with the photosynthetic capacity of leaves and contributes to plant biomass production (Wong *et al.,* 1979, Siebrecht *et al.,* 2003, Gleason *et al.,* 2021). In the case of Cu delivery, high rates of photosynthesis should require more plastocyanin (e.g., more Cu), and thus may require a higher stomatal conductance to facilitate Cu delivery to the most photosynthetically active leaves via the transpiration stream (xylem). Leaf age, a developmental variable, may also affect Cu delivery after deficiency in poplar (Ravet *et al.,* 2011, Shahbaz *et al.,* 2015). As poplar leaves develop, they undergo physiological, morphological, and structural changes, which should influence how nutrients are delivered to the sites of photosynthesis (Lawrence *et al.,* 2021). Interestingly, Lawrence *et al.,* (2021) found that leaf vein density increased with leaf position (leaf position 10 and 25) in hybrid poplar. Given that leaf hydraulic conductance often scales with vein density within and across species (Brodribb *et al.,* 2007, Boyce *et al.,* 2009), it is likely that variation in vein density would also be aligned with water and nutrient transport capacity (Lawrence *et al.,* 2021).

Furthermore, we know that leaf age can affect photosynthesis across species from *Arabidopsis* to peach trees (*Prunis persica*) (Bielczynski *et al.,* 2017, Marchi *et al.,* 2008). Bielczynski *et al.,* (2017) examined leaf age effects on photosynthesis in the developing rosette

of *Arabidopsis* using chlorophyll fluorescence imaging. These researchers measured leaf age in days after emergence and found that ΦPSII (electron flux through photosystem II, an indication of PSII operating efficiency) increased with leaf age. (Murchie and Lawson 2013, Bielczynski *et al.,* 2017). Marchi and coworkers (2008) observed that peach leaves increased their photosynthetic capacity once fully expanded which suggested that older leaves have larger photosynthetic capacity.

Before Cu can be delivered to sites of photosynthesis, it must be taken up into plants via the root system and transported through the vasculature (xylem or phloem) in stems to the leaves for cofactor assimilation (Burkhead *et al.,* 2009). Long distance transport of Cu in the vasculature is likely completed while bound to a stable complex such as the non-proteogenic amino acid nicotianamine (NA) or histidine (Ryan *et al.,* 2013). Cao and colleagues recently published stable isotope evidence that Cu could be mobile in the phloem of willow (*Salix integra* Thunb) for redistribution (Cao *et al.,* 2020). Stable isotopes can be used to quantify nutrient movement. Isotopes with low natural abundance can be supplied in pure form and then traced through a plant using mass spectrometry. The stable isotopes ^{63}Cu and ^{65}Cu are present at natural abundances of 69.15% and 30.85%, respectively (Savage 2018).

Our objective for this study was two-fold: (1) evaluate the use of an enriched Cu hydroponic solution (98% ^{65}Cu) to quantify Cu uptake and distribution directly from the hydroponic media (increase in ^{65}Cu) and movement out of old leaves (increase in ^{63}Cu). (2) examine how symptoms associated with Cu deficiency in the leaves evolve over time when plants are resupplied with Cu. To this aim we measured plant growth, leaf age, photosynthesis, stomatal conductance, and Cu isotope concentration. We aimed to answer the questions how Cu is delivered at the organ level, how is it distributed/partitioned across the whole plant, and what are the traits that affect its long-distance transport in poplar. We hypothesized that increases in ^{65}Cu will indicate the source of Cu translocation and reveal where Cu is prioritized to after resupply.

2.3 MATERIALS AND METHODS

Plant material and growth conditions

Hybrid white poplar (*P. tremula* x *P. Alba*, INRA 717-1B4) seedlings were propagated *in vitro* on ½ strength Murashige and Skoog (Sigma-Aldrich) hormone free media with sucrose (20 g/L) and agar (7 g/L). For hydroponic growth, seedlings were removed from the agar and the roots were washed with DI water. ~8 cm tall, rooted explants with an average age of 3 months were randomly distributed to 20-L black plastic buckets with an aerated one-tenth strength modified Hoagland's solution (3 plants per bucket) (Hoagland and Arnon, 1950; Shahbaz *et al.,* 2015). The hydroponic solution pH was adjusted to 5.9 with KOH (Shahbaz *et al.,* 2015). Seedlings were immediately covered with Magenta® boxes to maintain high humidity. The covers were gradually lifted and removed over the first 7 days. Plants were grown in a climate-controlled room under a light intensity of 150 µmol m^{-2} s^{-1}, with a 16h day and 8h night photoperiod. Temperature was maintained at 22°C±1°C. Cu sufficient treatments were given a 50 nM $CuSO_4$ natural isotopic content (69.17% ^{63}Cu and 30.83% ^{65}Cu) solution. Cu deficient treatments were the same as Cu sufficient treatments except $CuSO_4$ was omitted from the hydroponic solution. The control for the experiments in this study are the "+Cu" and the treatments are the "-Cu", "Pulse24", and "Pulse72".

Cu Isotope enrichment (Pulse) during resupply

After 6 weeks of Cu deficiency, plants from the Cu deficient buckets were placed in a 3L black plastic bucket (1 plant per bucket) with an aerated one-tenth strength modified Hoagland's solution (Shahbaz *et al.,* 2015). Plants were given a 50 nM $CuSO_4$ solution enriched in ^{65}Cu (98% ^{65}Cu and 2% ^{63}Cu) (Isoflex, San Francisco, CA) for 24 h (Pulse24) and 72 h (Pulse72). Growth conditions are described above.

Cu isotope quantification

Leaf and stem samples were oven dried at 50°C for 3 days. Dried tissue was digested in 1 mL of concentrated nitric acid and heated at 60°C for 2h followed by 130°C for 6h (Pilon-Smits *et al.,* 1999). Digests were diluted with deionized water to 10 mL and analyzed with a NexION 350D mass spectrometer in Quantitative Analysis mode (PerkinElmer, Waltham, MA). The Cu standard used was $CuSO_4$ in 3% HNO_3 (Inorganic Ventures, Christiansburg, VA). Iridium was used as the internal standard (QC).

Chlorophyll PAM fluorescence measurements

Leaf 3 from all treatments were excised between 6-8h in the photoperiod, the petioles placed in DI water, and dark-adapted for 15 min before chlorophyll fluorescence measurements with an FMS system with a leaf clamp (Hansatech, Norfolk, UK). Measurements were made in the center of one half of the leaf. The program used to estimate chlorophyll fluorescence parameters is detailed in Cohu and Pilon (2007). Actinic light intensities used were 230, 530, and 1250 µmol m^{-2} sec^{-1}. The following parameters were measured: photosystem II quantum efficiency (ΦPSII), photochemical quenching (qP), and non-photochemical quenching (NPQ) according to the equations detailed in Murchie and Lawson (2013).

Whole-plant chlorophyll PAM fluorescence imaging

Plants were dark adapted for 15 min before imaging. All work was done in a dark room under dim green light. Briefly, each plant was placed in an Erlenmeyer flask with 10% Hoagland's solution with 98% ^{65}Cu from the individual black buckets in the respective treatment (24 or 72 hours of resupply). Images were taken with a WALZ MAXI Imaging PAM (Effeltrich, Germany). The script contained a saturating pulse intensity of 10 s at a width of 840 ms to measure F_v/F_m followed by measurements of ΦPSII at actinic light intensities of 230, 530, and 1250 µmol m^{-2} sec^{-1}. Five to seven Areas of Interest (AOI) were selected for quantitative fluorescence measurements on visible leaves from the top of the canopy in between midribs and major veins (Osório *et al.,* 2014).

Plant Sampling and Height Measurements

The experiments performed in this study were done with at least three biological replicates. To quantify differences in isotope levels between young and mature leaves, leaf age was assigned by measuring the first leaf that was 2 cm long, designating it Leaf 1 (Larson and Isebrands, 1971). Young leaves (Leaf 0-2) and mature leaves (Leaf 3, Leaf 5, and Leaf 7) were excised with a razor blade at the base of the leaf. Leaves smaller than 2cm were given the designation “Leaf 0” (e.g., leaves growing out of the apical meristem). Leaf 5 and Leaf 7 were fully expanded in +Cu and -Cu treatments. +Cu plants have approximately 15-17 total leaves and -Cu plants have approximately 9-10 leaves. For chlorophyll fluorescence analysis, leaf 3 was excised at the petiole, placed in DI water, and dark adapted. Plant height was measured from the bucket lid to the apical meristem weekly.

Stomatal conductance (g_s) measurements

Stomatal conductance data was collected using a hand-held steady-state porometer (Model SC-1, Meter Group Inc). Stomatal conductance was measured on Leaf 3, 5, and 7 for the first six weeks in +Cu and -Cu treatments. It was then measured every 4 hours during the three day isotope enrichment period.

Statistical Analysis and Graphics

Figures were done using the “ggplott2” package (Wickham 2016). Analyses were done using R 4.0.3. (R Core Team 2020) and JMP software (version 15.0.0). One-Way ANOVAs were performed using the “lm” function and Tukey mean separation was done using the “emmeans” package (Length 2021) at a significance level of 0.05 (R Core Team 2020). Two-sample t-tests were performed with the “t.test” function with a significance level of 0.05 (R Core Team 2020). Two-Way ANOVAs were performed using JMP software (version 15.0.0).

2.4 RESULTS

Plant Growth, Cu deficiency, and symptomatic recovery

We followed the phenotypic spatial and temporal progression of Cu deficiency symptoms in leaves and observed how they recovered upon Cu resupply with 50 nM $CuSO_4$ (Figure 2.1). Several well-known Cu deficiency symptoms: stunted growth, leaf curling, and chlorosis along the midrib and in between major veins were first visible in week 5 and progressed into more severe symptoms in week 6 (Figure 2.1B and 2.1D). Compared to Cu-deficient plants, Cu-sufficient control plants appeared healthy and had more leaves, a faster leaf expansion rate, and thicker stems (Figure 2.1A and 2.1C, Figure 2.7). To observe phenotypic changes resulting from resupply, we transferred Cu deficient plants to 10% Hoagland's containing 50 nM $CuSO_4$ at the end of week 6. Chlorotic spots located next to the midrib and major veins regained chlorophyll after 24 hours of Cu resupply (Figure 2.1E). After 72 hours of resupply, leaves were noticeably greener around the midrib and around major veins compared to deficient leaves (Figure 2.1F). The recovery of the Cu deficiency induced leaf symptoms appeared partially complete by day 3, as expected (Shahbaz *et al.,* 2015).

Spatiotemporal and physiological effects of Cu deficiency on leaf-level physiology

+Cu plants and -Cu plants grew to the same height until approximately week 4 (Figure 2.2C). There were small and significant differences in how Cu was distributed amongst young and mature leaves in control and deficient conditions (Figure 2.2A). To visualize spatial and temporal heterogeneities in photosynthesis in young and mature leaves, we imaged chlorophyll fluorescence in +Cu and -Cu plants from 4, 5, and 6 weeks of growth (Figure 2.2B, Figure 2.9-2.11). It was found that ΦPSII was significantly higher in the +Cu plants in weeks 4, 5, and 6 (Table 2.2). We observed based on the images that mature leaves had higher ΦPSII (an indication of PSII operating efficiency) than younger leaves in weeks 4, 5, and 6 in +Cu plants (Figure 2.2B, Figures 2.9-2.11). The -Cu leaves had very low ΦPSII in between major veins, but the older leaves in the middle of the canopy showed slightly higher ΦPSII overall compared to younger leaves (Figure 2.2B). ΦPSII along the midrib and major veins of older leaves was slightly higher in -Cu plants as well (Figure 2.2A). To investigate a trait to represent water

transport in leaves, we measured stomatal conductance. Maximal stomatal conductance represents the water transport capacity per unit leaf area via the xylem, normalized by vapor pressure deficit (Monteith 1965). Stomatal conductance was significantly higher in +Cu leaves from weeks 4 to 6, but not in week 3 (Figure 2.2D-G).

Photosynthetic recovery upon Cu resupply

Chlorophyll fluorescence parameters were measured on Leaf 3 from all treatments to assess the recovery of electron transport as Cu re-entered the leaf. Leaf 3 was chosen across both treatments due to the destructive sampling and script time of the Hansatech FMS system. The parameter F_v/F_m indicates PSII maximum capacity. There were only slight and non-significant differences in F_v/F_m between the four treatments (+Cu, -Cu, Pulse24, and Pulse72) (Table 2.1), indicating that PSII remained largely intact during the Cu deficiency treatment and subsequent resupply. As Cu enters the leaf during resupply, ΦPSII should increase as electron transport is restored. Prior to Cu resupply, ΦPSII in +Cu plants was higher compared to Cu deficient plants (Leaf 3) (Figure 2.3A). When 50 nM $CuSO_4$ was given to Cu deficient plants, ΦPSII values increased after 24 h slightly above –Cu values under low and mid light intensity (Figure 2.3A). However, 72 h after Cu resupply, ΦPSII had nearly returned to the control level (Figure 2.3A). NPQ (non-photochemical quenching) represents the dissipation of excess excitation energy in PSII (Murchie and Lawson 2013). As Cu becomes incorporated into PC, linear electron flow increases resulting in a steeper proton gradient across the thylakoid membrane (Murchie and Lawson 2013). Therefore, the protective capacity of NPQ can be affected by Cu availability in the leaf for electron transport (Murchie and Lawson 2013, Shahbaz *et al.,* 2015). Cu availability affects both 1-qP (redox state of the plastoquinone pool) and for NPQ (non-photochemical quenching) parameters (Figure 2.3B and 2.3C). The close alignment between fluorescence measurements and the timing of Cu resupply demonstrates the critical dependency of PSII function on Cu availability.

Spatiotemporal Cu partitioning in tissues, photosynthetic recovery, and changes in stomatal conductance (g_s) upon pulse with ^{65}Cu

Here, we supplied roots of Cu-deficient poplar with a pulse of ^{65}Cu, the less-abundant stable Cu isotope, to measure Cu movement at the whole-plant level from root to shoot and within the shoot. Additionally, we used chlorophyll fluorescence imaging of whole plants to provide a visualization of the photosynthetic response of Cu resupply in leaves. Overall, young leaves acquired ~58% more ^{65}Cu on day 1 (Pulse24) and ~65% more ^{65}Cu by day 3 (Pulse72) of resupply, compared to mature leaves (Figure 2.4A). In leaves, we observed increases in ^{63}Cu in all time points, but they did not rise after 24 h of resupply (Figure 2.4A). Only young stems showed a ~36% increase in ^{63}Cu after 24 h of resupply (Figure 2.4B). Stems acquired higher levels of ^{65}Cu compared to leaves with a noted increase between 24 h and 72 h (Figure 2.4B). There were no significant differences in leaf or stem age effect on Cu isotopes, but there were significant differences in treatment (time) effects on isotope levels (Table 2.4). Individual organ changes also show significant differences between treatment effect on Cu isotope levels (Figure 2.8). PAM images show an overall (qualitative) increase in ΦPSII upon pulse with ^{65}Cu (Figure 5A, Figure 2.12-2.15). This uptake of ^{65}Cu into the leaves of Cu deficient plants, and particularly the young leaves of these plants, resulted in a quick and marked recovery of ΦPSII (Figure 2.5A, Figure 2.12-2.15). On average, ΦPSII was higher in leaves supplied with ^{65}Cu and significantly different between -Cu, Pulse24, and Pulse72 treatments (Table 2.3). Furthermore, the recovery of ΦPSII was also not uniform across the leaf surface, with the lamina between the primary and secondary veins exhibiting more rapid and complete recovery (Figure 2.5A, Figure 2.14-2.15). Maximal g_s was higher in Leaf 5 and Leaf 7 than in Leaf 3, but only within the first 24 hours of isotope enrichment (not significant) (Figure 2.5B, Table 2.5). By ca day 2 after resupply, Leaf 3 began to exhibit higher g_s than Leaf 5 and Leaf 7 and continued to do so until the end of the 72 h enrichment period although there were no significant differences found

between leaf age and g_s (Figure 2.5B, Table 2.5). There were significant differences in time and g_s (Table 2.5).

2.5 DISCUSSION

Our objectives for this study were to determine if Cu isotopes (^{63}Cu and ^{65}Cu) can be used to quantify Cu prioritization and partitioning, and whether leaf and whole-plant level traits correlate with these processes upon Cu resupply. The speedy recovery of ΦPSII in Cu deficient plants corresponded closely with Cu uptake directly from the enriched hydroponic solution and appeared be complete 3 days after enrichment (Figure 2.1-2.3). We also found that Cu delivery was prioritized to the young leaves and stem (higher ^{65}Cu) compared to mature leaves (higher ^{63}Cu). There was also some Cu translocation from older tissues (higher ^{63}Cu) into young leaves (Figure 2.4).

Spatiotemporal and physiological effects of Cu deficiency on leaf-level physiology

Photosynthetic performance began to decline in the leaf at week 4 and progressed through week 6 with Cu deficiency having little effect Cu distribution (Figure 2.2A-2.2C). Imaging PAM results suggest a decline in photosynthesis in the mesophyll in between the midrib and higher order veins (lower ΦPSII) from leaves in the upper portion of the plants (Figure 2.2A, Figures 2.9-2.11). The higher ΦPSII in mature leaves of +Cu poplar suggests they remained more photosynthetically active and may have functioned as source leaves, supplying Cu via the phloem to the top 1-3 leaves (Figure 2.4B). The low ΦPSII in -Cu leaves in week 4 were only present in the upper portion of the canopy, suggesting older leaves lower in the canopy still had sufficient Cu to maintain a higher PSII operating efficiency (Figure 2.2B). This could also mean that Cu was not translocated efficiently during Cu deficient conditions. More specifically, it could be that mature leaves do not share their Cu with young leaves during deficiency even if they are carbon sources. Since environmental cues have been suggested to be integrated with Cu homeostasis, we wanted to know how Cu deficiency affected stomatal function (Zhang *et al.,*

2014, Yan *et al.,* 2017, Rahmati *et al.,* 2020). Stomatal conductance was measured as soon as the first new leaves began to appear after treatments were applied. When comparing +Cu and -Cu plants, g_s at week 3 probably represents similar plant performance due to there being no visual symptoms of Cu deficiency in week 3 (Figure 2.2C).

Spatiotemporal Cu partitioning in tissues, photosynthetic recovery, and changes in stomatal conductance (g_s) upon pulse with ^{65}Cu

Isotope measurements confirm prioritization for Cu delivery for the young leaves in poplar as reported before (Shahbaz *et al.,* 2015). The new Cu that is in young leaves and stems could have two possible sources: transport from the hydroponic solution via the xylem or redistribution among tissues and organs via the phloem. The speed of ^{65}Cu enrichment in tissues (within 72 hours) strongly suggest that the ^{65}Cu was taken up directly from the hydroponic solution (Figure 4) (Shahbaz *et al.,* 2015).

Leaf age also seems to have a partial role on Cu distribution in poplar (Figure 2.4). Shahbaz *et al.,* (2015) observed that Leaf 0-2 received more Cu than Leaf 3-9 five days after resupplying Cu. Considering only ^{65}Cu content in leaves, our data suggests that leaf age plays a partial role in where Cu is delivered and the majority of ^{65}Cu was partitioned to the stem (Figure 2.4). Furthermore, the ^{65}Cu in the stem could represent Cu that is in the xylem vessels being transported to the young leaves making use of the organ as a highway (Figure 2.4C). It could be that Cu partitioned is split between leaf photosynthesis (e.g., e^- transport) and utilization or storage in the stem, although it is unclear what stem functions would require a large fraction of Cu besides lignin polymerization in the cell wall (Bernal *et al.,* 2012). Our results also align with Cao et al (2020). Cao et al. (2020) reported that Cu can be remobilized in willow (*Salix integra*.) from older leaves to younger leaves. This was also the first report on long-distance Cu transport in a tree species using stable Cu isotopes (Cao *et al.* 2020). Waters and Grusak (2008) demonstrated that mineral content fluctuates in vegetative and reproductive tissues throughout the life cycle of *Arabidopsis thaliana*. The Cu concentration was shown to decrease in leaf

tissue over time in wild type lines (Waters and Grusak 2008). These results from *Arabidopsis* could be aligned with what has been recently published in willow where Cu was shown to be translocated via the phloem (Cao *et al.,* 2020). An additional interesting, but not significant result was that older leaves exhibited higher stomatal conductance than younger leaves during the first day of Cu resupply, but after this point, g_s increased in younger leaves (Figure 2.5). These results could indicate the prioritization of younger leaves during recovery, given that higher stomatal conductance could possibly result in more Cu being transported to young leaves via the xylem.

Chlorophyll fluorescence imaging revealed younger leaves were markedly affected by Cu deficiency along the midrib and major veins (Figure 2.5A). However, imaging also revealed that leaf tissue closer to major veins recovered ΦPSII function faster than tissue located more distally from major veins (Figure 2.5A). This may indicate that Cu is being delivered from the vasculature through the major veins first (Figure 2.5A). Although the isotope data suggests that ^{65}Cu was delivered from the hydroponic media, ^{63}Cu was also redistributed from older tissue (Figure 2.4). Taken together, there seemed to be some correlation between g_s, isotope, and whole-plant fluorescence data that suggests that leaf age is a top priority for Cu after resupply. Statistical results suggest that time has a more significant effect on ^{65}Cu resupply and stomatal conductance change upon ^{65}Cu resupply than leaf age (Figure 2.4; Table 2.4; Table 2.5). Figure 2.6 represents a whole-plant physiological response of pulse with 98% ^{65}Cu. Isotope colors represent the shifts seen in leaves and stems (Figure 2.4A and 2.4B).

2.6 CONCLUSIONS

In conclusion, by looking at the movement of both isotopes, we can conclude that a large fraction of Cu influx in young leaves is coming from the hydroponic media with some ^{63}Cu possibly coming from older tissues. Cu delivery to photosynthesis is prioritized by leaf age. These results may lead to a quantitative understanding of how Cu is systemically transported

and distributed to photosynthetic and stem tissues in woody angiosperms. Data on stomatal conductance suggests a fast recovery of xylem mediated Cu transport to younger leaves during Cu resupply, which should help to deliver Cu where it is most needed in the plant.

2.7 FIGURES AND TABLES

Table 2.1. F_v/F_m of +Cu, -Cu, and Cu resupplied plants after 24 and 72 h. Values represented as mean ± 1SE. +Cu (n=3) -Cu, Pulse 24, Pulse 72 (n=4). F_v/F_m from the FMS system.

Treatment	F_v/F_m
+Cu	0.83 ± 0.002
-Cu	0.79 ± 0.007
Pulse 24	0.78 ± 0.011
Pulse 72	0.79 ± 0.019

Table 2.2. ΦPSII in +Cu and -Cu treatments. Values represented as mean ± 1SE. (n=18). The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. (t-test). ΦPSII from Imaging PAM

Week	Treatment	ΦPSII
Week 4	+Cu	0.190 ± 0.012***
	-Cu	0.0866 ± 0.006***
Week 5	+Cu	0.159 ± 0.010***
	-Cu	0.0531 ± 0.006***
Week 6	+Cu	0.184 ± 0.010***
	-Cu	0.055 ± 0.006***

Table 2.3. ΦPSII in +Cu, -Cu, Pulse24, and Pulse72 treatments. Values represented as mean ± 1SE. (n=6). Letters represent Tukey adjusted p-values (<0.05). ΦPSII from Imaging PAM.

Treatment	ΦPSII
+Cu	0.143 ± 0.072
-Cu	0.070 ± 0.075[a]
Pulse 24	0.207 ± 0.032[a]
Pulse 72	0.227 ± 0.028[a]

Table 2.4. Results of two-way ANOVA for the effects of treatment and leaf age and the interaction of these effects on isotope levels. The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Isotope or Organ	Leaf Age	Treatment	Leaf age x Treatment
Leaf ^{63}Cu	$p = 0.756$	***	$p = 0.900$
Leaf ^{65}Cu	$p = 0.184$	***	$p = 0.236$
Stem ^{63}Cu	$p = 0.227$	***	*
Stem ^{65}Cu	$p = 0.628$	***	$p = 0.378$

Table 2.5. Results of two-way ANOVA for the effects of time after resupply and leaf age and the interactions of these effects on g_s. The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

	Leaf Age	Time (h)	Leaf age x Time
g_s	$p = 0.956$	***	$p = 0.062$

Figure 2.1. Symptomatic recovery of poplar after Cu resupply. A. +Cu plant at week 5. **B.** –Cu plant at week 5. **C.** +Cu plants at Week 6. **D.** –Cu plant at week 6. **E.** Plant after 24 h in 50 nM $CuSO_4$ (98% ^{65}Cu). **F.** Plant after 72 h in 50 nM $CuSO_4$ (98% ^{65}Cu). White arrows indicate phenotypes of interest: a) interveinal chlorosis, b) leaf margin curl c) regain of chlorophyll.

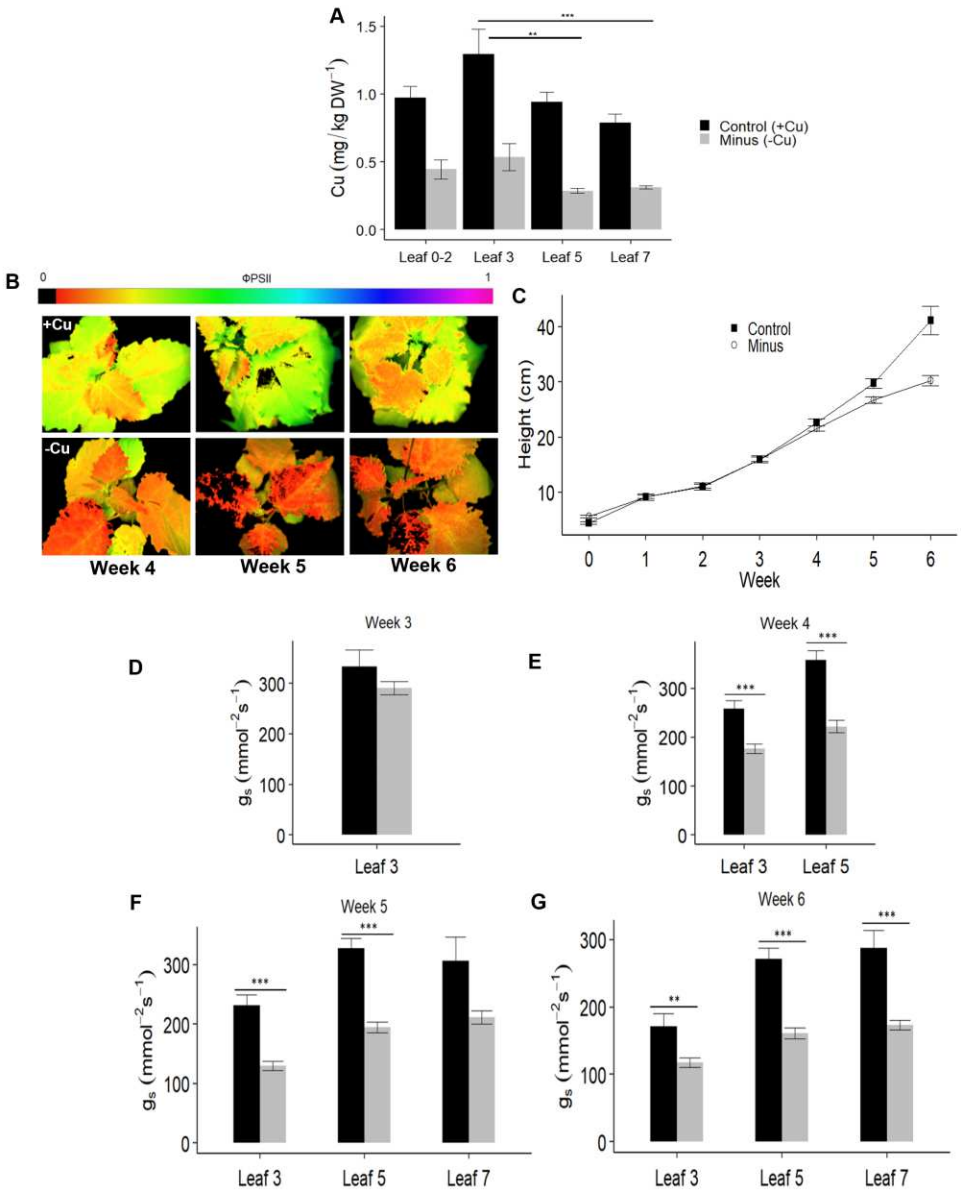

Figure 2.2. Spatiotemporal progression of plant growth, ΦPSII, Cu distribution, and stomatal conductance (g_s) in +Cu and -Cu poplar. A. Cu distribution in young and mature leaves. Values represented as mean ± 1SE (n=3). One-Way ANOVA with Tukey adjusted p-values indicate leaf age differences. The asterisks denote significant effects; ** = $P < 0.01$, *** = $P < 0.001$. **B.** Top view of ΦPSII in young and mature leaves in weeks 4, 5, and 6 of growth. +Cu (n=3). –Cu (n=3). **C.** Plant growth of +Cu and -Cu plants. +Cu (n=6). –Cu (n=18). Mean ± 1SE. **D-G.** Stomatal conductance in week 3-6 of +Cu and -Cu treatments. Values represented as mean ± 1SE. +Cu (n=6) –Cu (n=18). Asterisks represent significant differences (t-test, p<0.05).

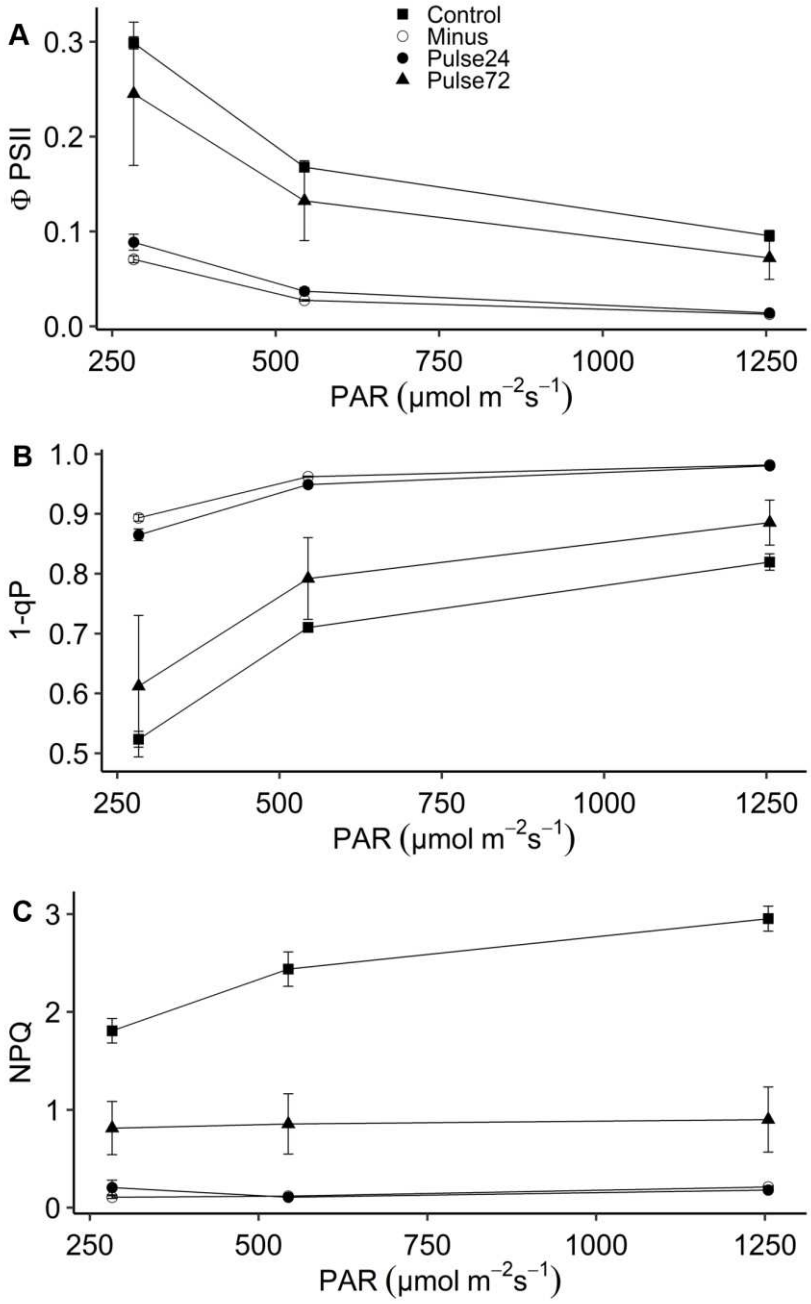

Figure 2.3. Photosynthetic recovery of hybrid white poplar upon Cu resupply. A. The quantum efficiency of PSII (ΦPSII) in Leaf 3 as a function of light intensity (Photosynthetically Active Radiation or PAR). **B.** 1 – qP, representing the redox state of the plastoquinone pool as a function of PAR for Leaf 3. **C.** Non-Photochemical Quenching (NPQ) for Leaf 3 as a function of PAR. ΦPSII, 1-qP, and NPQ were analyzed using an FMS system. *Closed squares*: control + Cu, *open circles:* – Cu plants, *closed circles:* 24 h Cu resupply (pulse 24), *closed triangles:* 72 h Cu resupply (pulse 72). Values are represented as mean ± 1SE. +Cu (n=3), -Cu, Pulse24, Pluse72 (n=4).

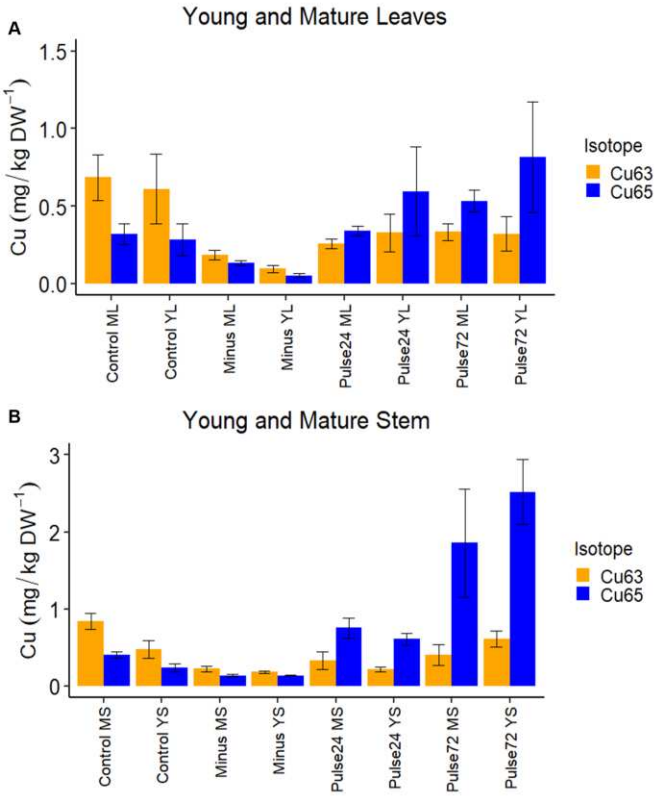

Figure 2.4. Cu movement in leaves and stems upon pulse with 98% ^{65}Cu. A. Isotopic concentration in young leaves. (n=6) and mature leaves. (n=18) **B.** Isotopic concentration in young stem. +Cu, -Cu, Pulse24 (n=6), Pluse72 (n=5) and mature stem. +Cu, -Cu, Pulse24 (n=6), Pluse72 (n=5). *YL:* Young Leaves. *ML:* Mature Leaves. *YS:* Young Stem. *MS:* Mature Stem. Values are shown as mean ± 1SE.

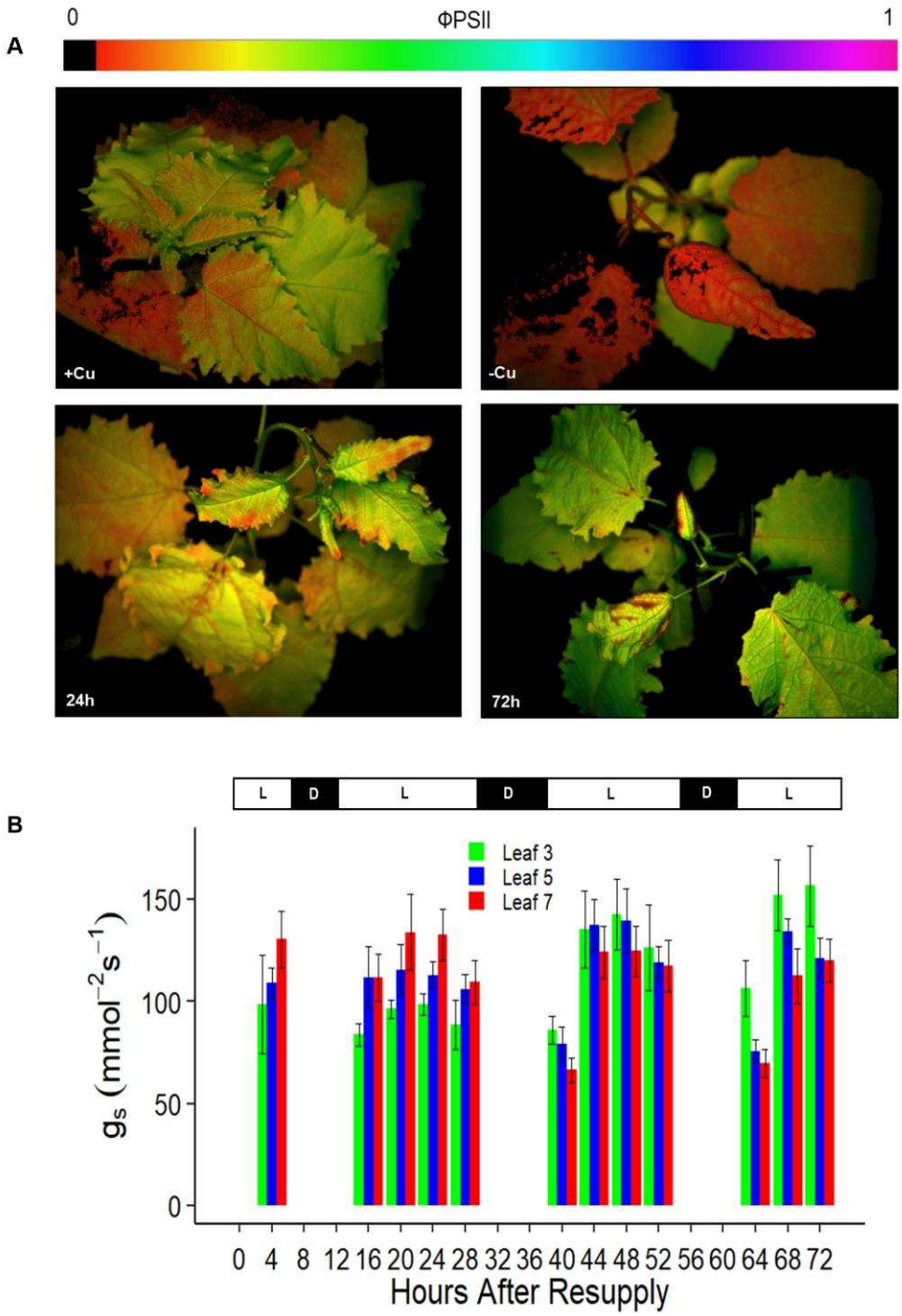

Figure 2.5. Spatiotemporal recovery from Cu deficiency of ΦPSII in leaves and stomatal conductance (g_s) upon Cu resupply (50 nM $CuSO_4$ pulse with ^{65}Cu). A. Top view of ΦPSII in young and mature leaves of +Cu, -Cu, Pulse24, and Pulse72 PAR: 230µmol m^{-2} sec^{-1}. **B.** g_s during the 72 hour enrichment. White bars indicated time in the light. Black bars indicate dark period. Values represented as mean ± 1SE (n=6).

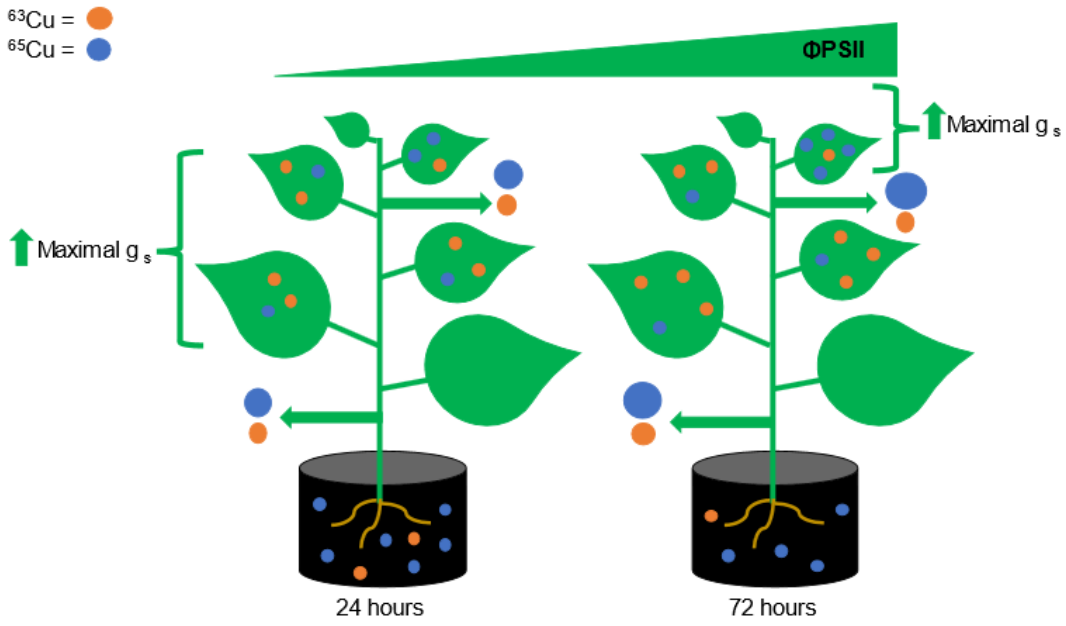

Figure 2.6. Conceptual model of plant response to 24 h and 72 h of ^{65}Cu enrichment. Isotope concentration shifts are represented in each leaf with number of dots in leaves and size of dots for stem data. g_s shifts are displayed by green brackets. Photosynthetic recovery via the Imaging PAM is represented by the triangle shape increasing from left to right above the plants.

Figure 2.7. Images of +Cu and -Cu plants. A. +Cu plants at week 5 of growth. **B.** -Cu plants at week 5 of growth. **C.** +Cu plants at week 6. **D.** -Cu plants at week 6. Plants shown here represent plant morphology before pulse is applied.

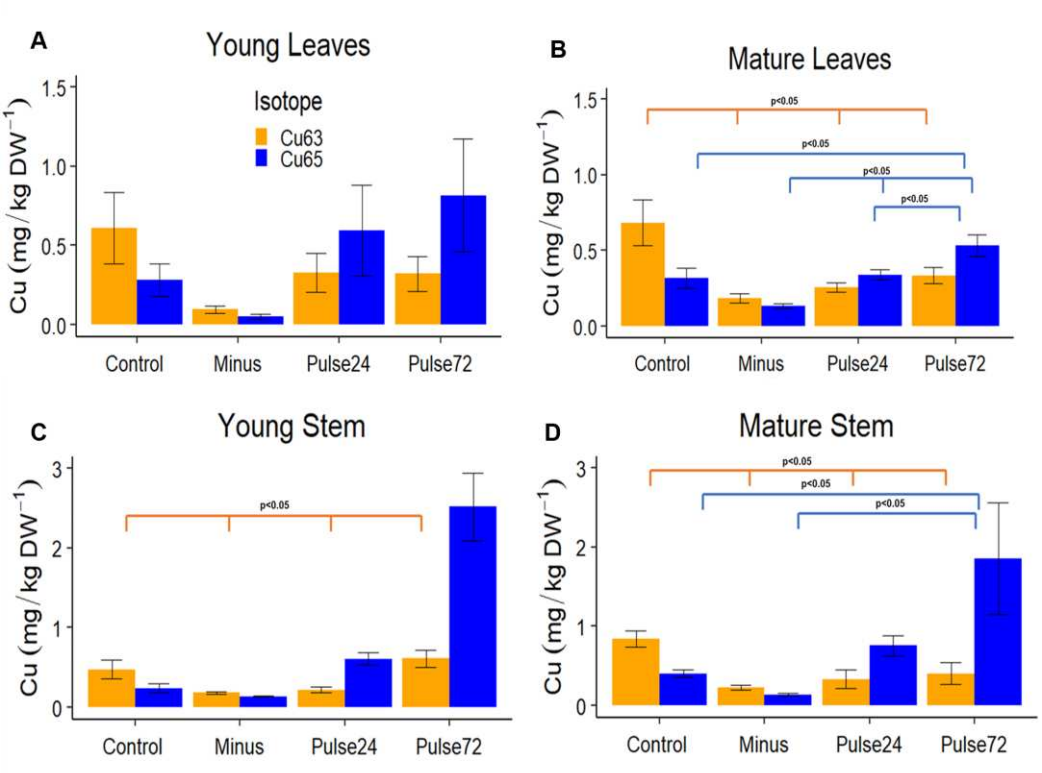

Figure 2.8. Cu movement in leaves and stems. A. Isotopic concentration in young leaves. (n=6) **B.** Isotopic concentration in mature leaves. (n=18) **C.** Isotopic concentration in young stem. +Cu (n=6), -Cu, Pulse24, Pluse72 (n=5-6). **D.** Isotopic concentration in mature stem. +Cu (n=6), -Cu, Pulse24, Pluse72 (n=5-6). Values are shown as mean ± 1SE. One-Way ANOVA results with significance are indicated with bars corresponding to isotope colors.

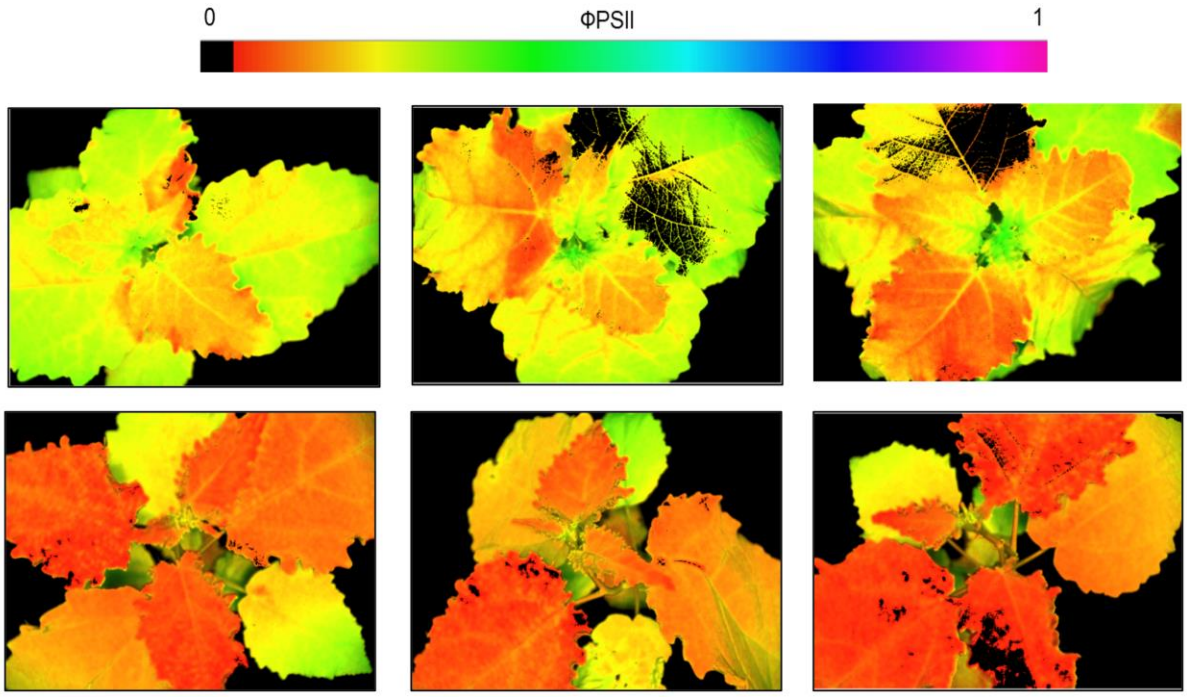

Figure 2.9. Top view of ΦPSII in young and mature leaves of +Cu and -Cu plants at 4 weeks of growth. PAR: 230µmol m^{-2} sec^{-1}. Top panel: +Cu plants. Bottom panel -Cu plants.

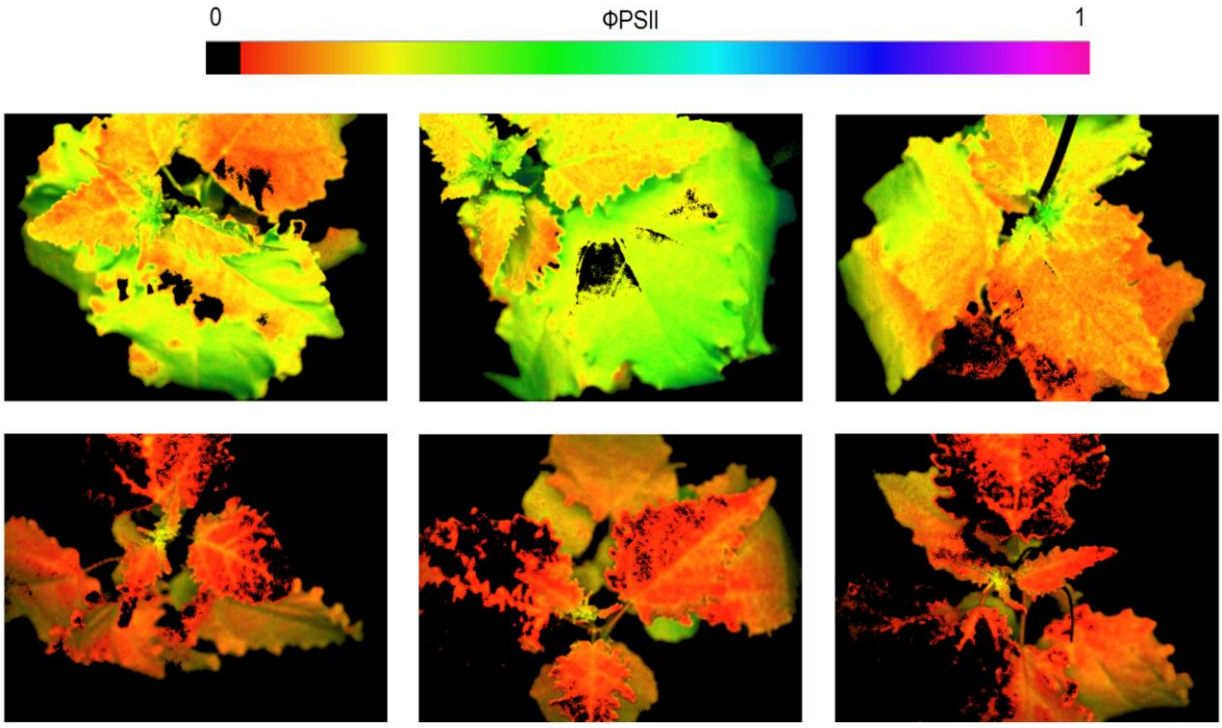

Figure 2.10. Top view of ΦPSII in young and mature leaves of +Cu and -Cu plants at 5 weeks of growth. PAR: 230µmol m^{-2} sec^{-1}. Top panel: +Cu plants. Bottom panel -Cu plants.

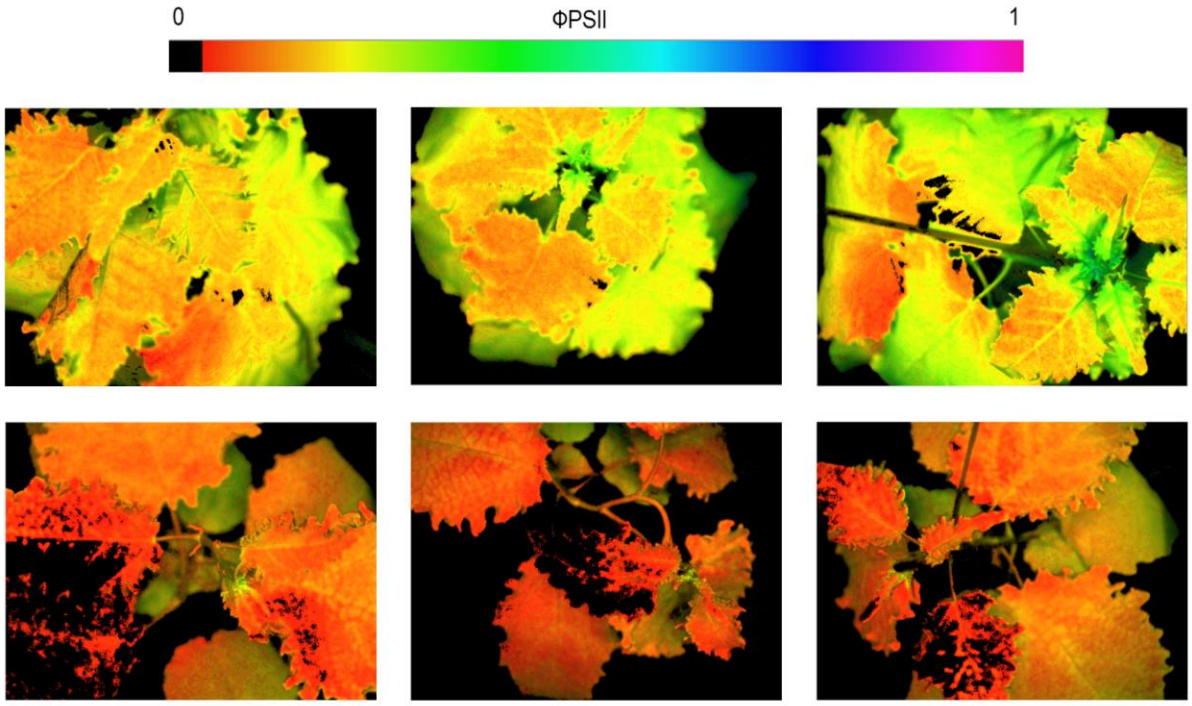

Figure 2.11. Top view of ΦPSII in young and mature leaves of +Cu and -Cu plants at 6 weeks of growth. PAR: 230µmol m^{-2} sec^{-1}. Top panel: +Cu plants. Bottom panel -Cu plants.

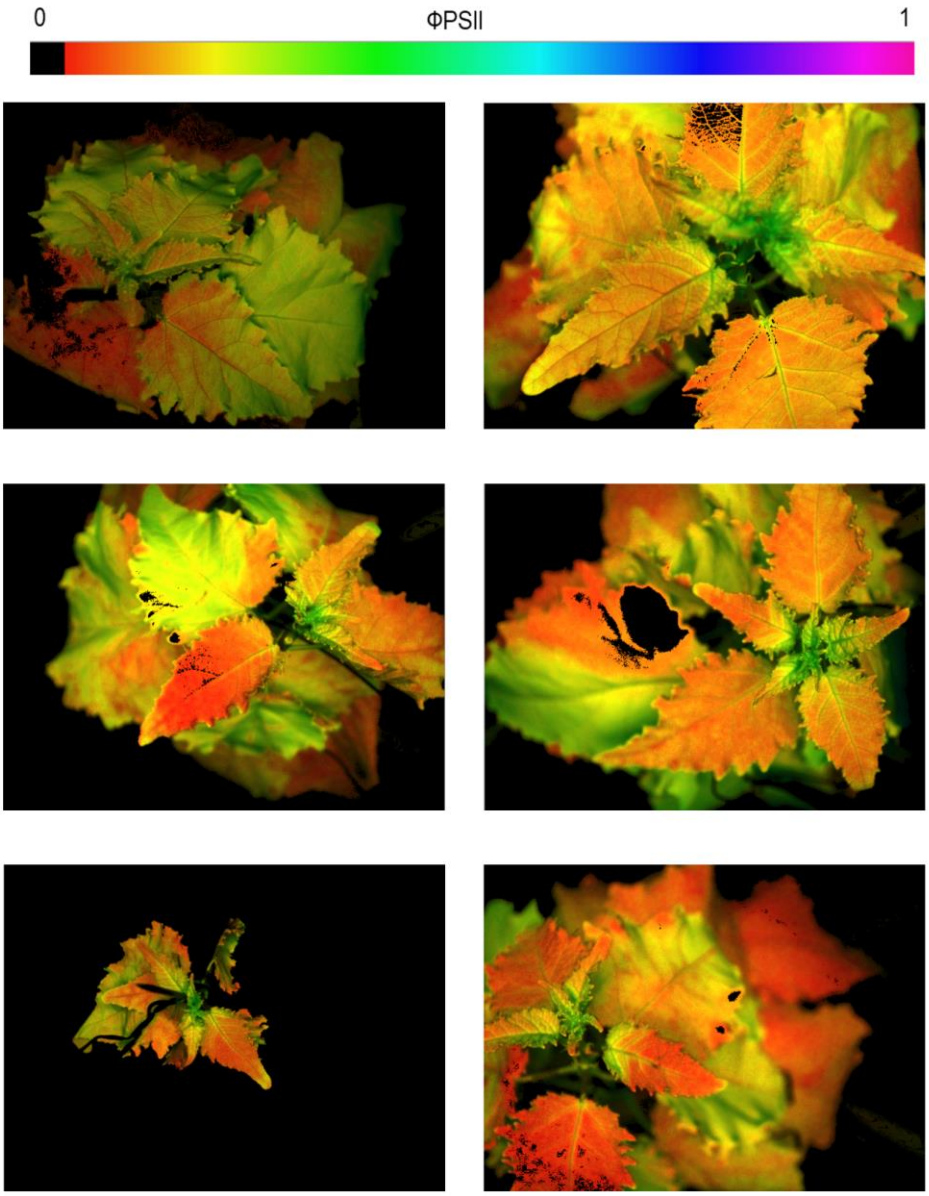

Figure 2.12. Top view of ΦPSII in young and mature leaves of +Cu plants at 6 weeks of growth for imaging PAM/pulse experiments. PAR: 230µmol m^{-2} sec^{-1}.

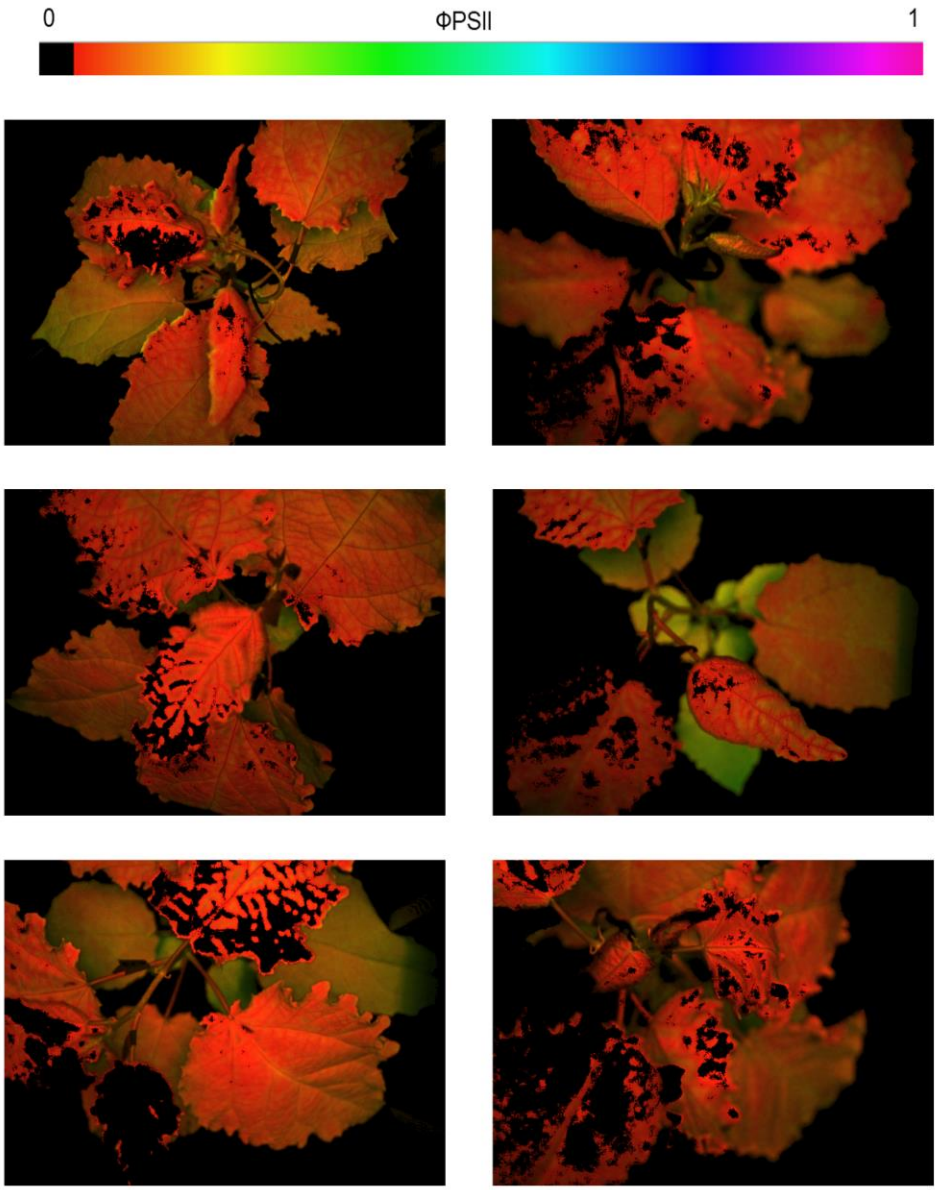

Figure 2.13. Top view of ΦPSII in young and mature leaves of -Cu plants at 6 weeks of growth for imaging PAM/pulse experiments. PAR: 230µmol m^{-2} sec^{-1}.

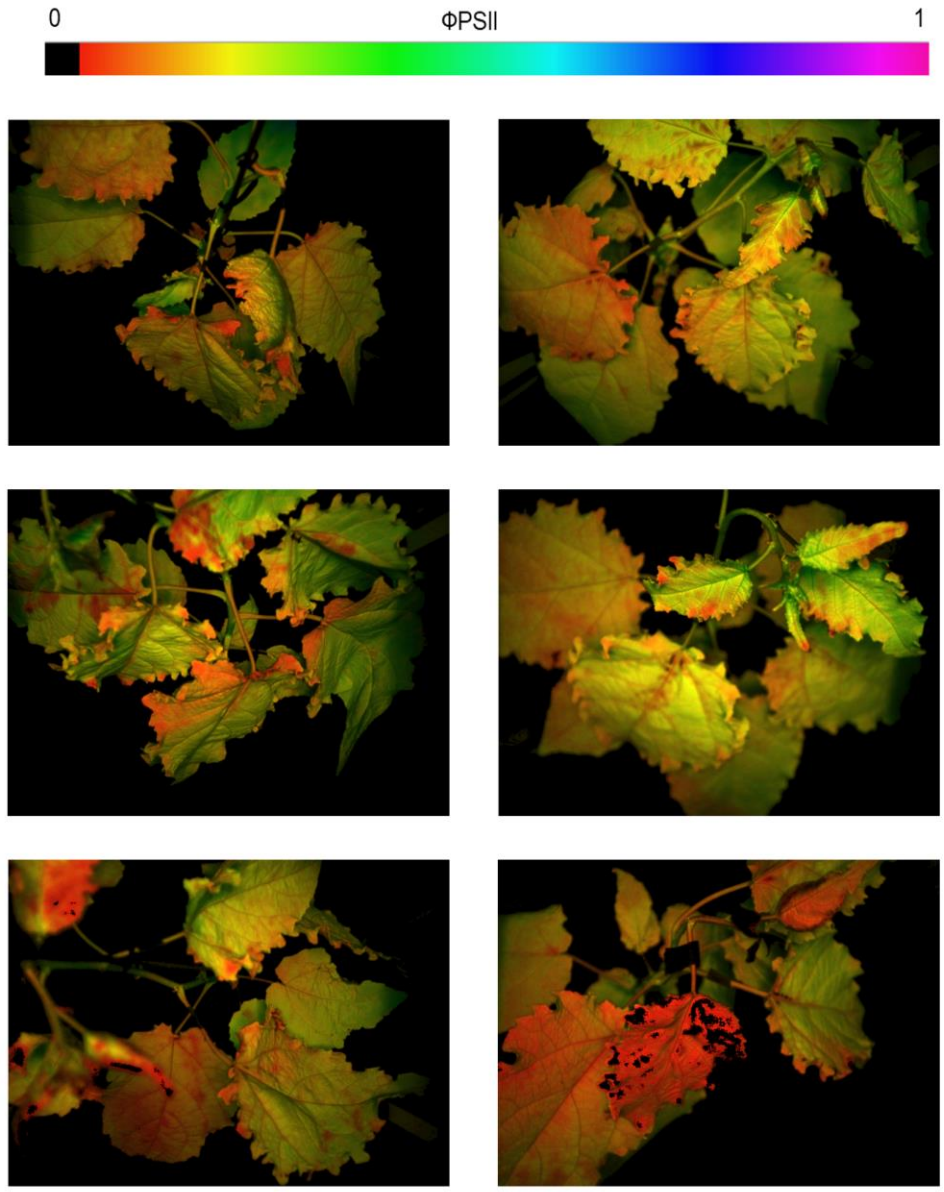

Figure 2.14. Top view of ΦPSII in young and mature leaves plants at 24 h in 50 nM $CuSO_4$ (98% ^{65}Cu). PAR: 230µmol m^{-2} sec^{-1}.

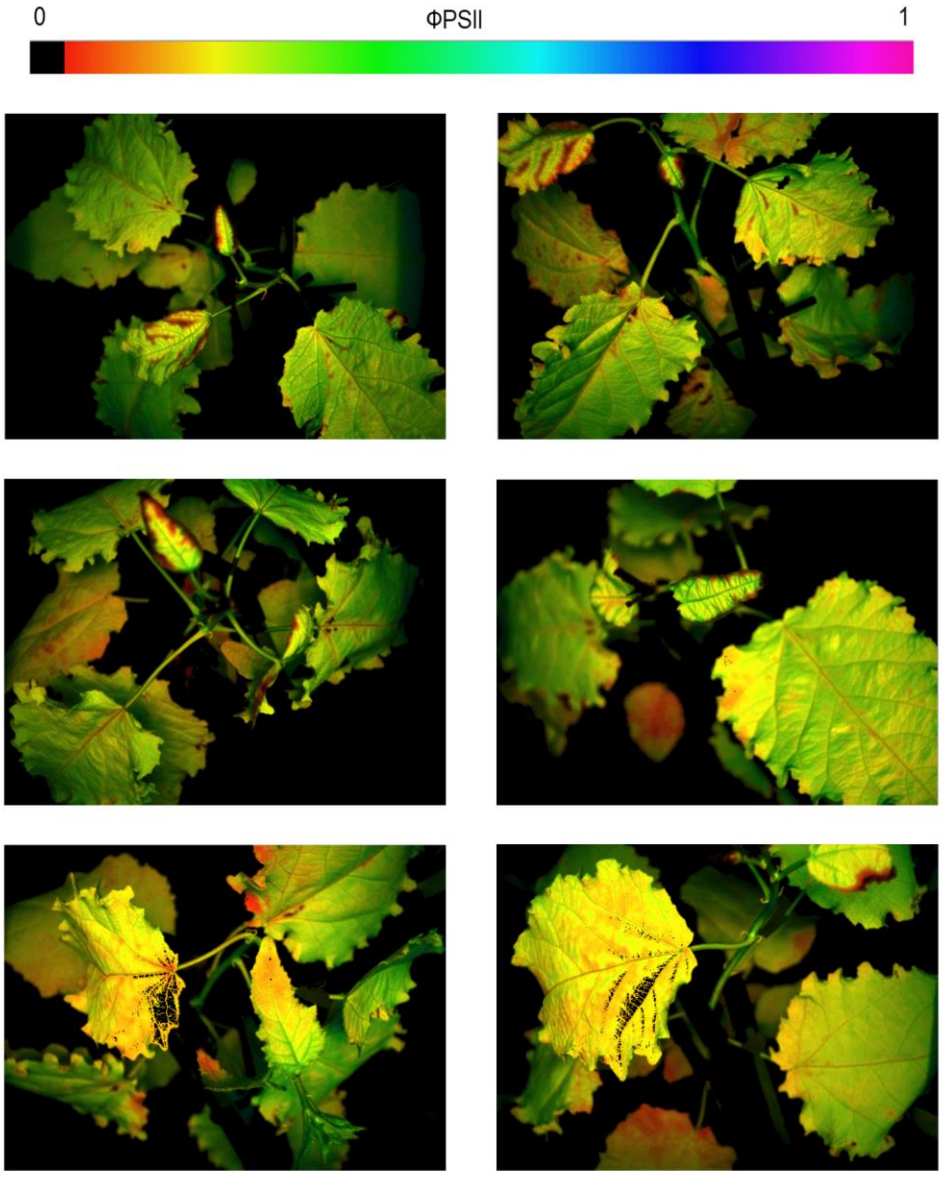

Figure 2.15. Top view of ΦPSII in young and mature leaves plants at 74 h in 50 nM $CuSO_4$ (98% ^{65}Cu). PAR: 230µmol m^{-2} sec^{-1}.

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CHAPTER 3: ORGAN AGE DEPENDENT DISTRIBUTION OF ESSENTIAL NUTRIENTS INDUCED BY COPPER FEEDING STATUS IN POPLAR[2]

3.1 SUMMARY

Copper (Cu) is an essential micronutrient, and its deficiency can cause plants to undergo metabolic changes at several levels of organization. Additionally, it has been shown that leaf age can play a role in nutrient distribution along the shoot axis of poplar. In this study, we investigated the effect of Cu deficiency on the altered distribution of essential macro and micronutrients in leaves and stems of different age. Our results indicate Cu deficiency caused higher mineral concentrations of Ca, Mg, S, Fe, Zn, Mn, and Mo in leaves and Ca, P, Fe, and Zn in stems, when compared to +Cu organs. Two-way ANOVA analyses revealed that in most cases leaf and stem age had significant effects on nutrient distribution. Principal Component Analysis revealed distinct clusters of elements whose concentrations were significantly altered by Cu deficiency (Mn, Mg, S, Mo, and Zn). To investigate possible Cu reallocation, we carried out Cu resupply experiments using isotope enrichment (98% ^{65}Cu) in Cu deficient plants. These experiments showed that some Cu was remobilized (change in ^{63}Cu) and there was preferential partitioning of Cu to young and mature stem tissues (increase in ^{65}Cu). These results suggest that Cu deficiency and developmental stage can significantly influence the distribution and homeostasis of macro and micronutrients in poplar tissues. Secondly, they also demonstrate the variability in magnitude of preferential allocation to different aerial tissues.

3.2 INTRODUCTION

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Mineral nutrients are needed in varying amounts by plant species and can be classified as macro- or micronutrients. Macronutrients: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) are needed at 1000 mg kg^{-1} DW (ppm) or higher (Marschner 2012; Epstein *et al.,* 2005). Micronutrients: copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn) are needed at 0.1-100 ppm (Marschner 2012; Epstein *et al.,* 2005). A review of nutrient utilization can be found in Marschner (2012).

Lack in essential nutrients can cause deficiency symptoms in plant tissues but can also trigger metabolic remodeling at several levels of organization (Marschner 2012; Billard *et al.,* 2014; Shahbaz *et al.,* 2015). For instance, when copper (Cu) metal becomes deficient a response is triggered at the molecular level which is referred to as the Cu economy system (Ravet *et al.,* 2011; Shahbaz *et al.,* 2015). Essentially, once Cu limitation is sensed, the transcription factor SPL7 is turned on (Bernal *et al.,* 2012). SPL7 turns on the expression of several microRNAs (Cu-miRNAs) that serve to mediate the degradation of mRNAs encoding for target Cu proteins (Yamasaki *et al.,* 2009). Furthermore, the available Cu pool is hypothesized to be utilized in plastocyanin to continue photosynthetic electron transport (Shahbaz *et al.,* 2015). While metabolic remodeling after Cu deficiency has been shown to occur at the molecular level by affecting gene expression, Cu nutrient deficiency can also affect allocation of other essential nutrients in plant organs. The demand for nutrients during plant growth can be governed by many environmental and physiological factors. This demand can vary greatly along the axis of the shoot and differ depending on the nutrient, organ, and developmental stage under consideration (Siebrecht *et al.,* 2003). For instance, it has been shown that the demand for Nitrogen is much higher in young leaves versus old leaves (Gonzalez-Real and Baille 2000).

Given the different demand for elements of various plant organs we were interested to investigate how Cu deficiency affects nutrient allocation in different plant parts, including the mobility of Cu itself. Given the body of evidence that suggests crosstalk between Cu homeostasis and Fe, Zn, and Mo homeostasis (Waters and Armbrust 2013; Carrió-Seguí *et al.,*

2019; Saenchai *et al.,* 2016; Kuper *et al.,* 2004) it can be hypothesized that Cu deficiency will alter the nutrient distribution especially for Mo, Fe, and Zn. The objective for this study was two-fold: (1) to examine how Cu deficiency altered the distribution of mineral macronutrients (Ca, Mg, P, K, S) and micronutrients (Fe, Zn, Mn, and Mo) in leaves and stems of different age in hybrid poplar. (2) to examine mobility and remobilization of Cu itself in Cu deficient plants via stable isotope tracing.

3.3 METHODS

Plant material and growth conditions

Hybrid white poplar (*Populus tremula* × *P. alba*, INRA 717-1B4) seedlings were propagated *in vitro* on ½ strength Murashige and Skoog (Sigma-Aldrich) hormone free media with sucrose (20 g/L) and agar (7 g/L). For hydroponic growth, seedlings were removed from the agar and the roots were washed with DI water. Explants that were rooted and ~8 cm tall, rooted explants with an average age of 3 months were randomly distributed to 20-L black plastic buckets with an aerated one-tenth strength modified Hoagland's solution (3 plants per bucket) (Hoagland and Arnon, 1950; Shahbaz *et al.,* 2015). The hydroponic solution pH was adjusted to 5.9 with KOH (Shahbaz *et al.,* 2015). Seedlings were immediately covered with Magenta® boxes to maintain high humidity. The covers were gradually lifted and removed over the first 7 days. Plants were grown in a climate-controlled room under a light intensity of 150 µmol m^{-2} s^{-1}, with a 16h day and 8h night photoperiod. Temperature was maintained at 22°C±1°C. Cu sufficient treatments were given a 50 nM $CuSO_4$ natural isotopic content (69.17% ^{63}Cu and 30.83% ^{65}Cu) solution. Cu deficient treatments were the same as Cu sufficient treatments except $CuSO_4$ was omitted from the hydroponic solution. The control for the nutrient distribution experiments in this study are "+Cu" and the treatment is "-Cu". The pulse experiments are as follows: control "+Cu", treatments: "-Cu" "Pulse24", and "Pulse72". In our -Cu treatment, Leaf 7

is approximately the first leaf to fully develop out of tissue culture. Young stems were parts of stems above leaf 7 and mature stems were considered to be the parts of stems below Leaf 7.

Cu Isotope enrichment (Pulse) during resupply

After 6 weeks of Cu deficiency, plants from the Cu deficient buckets were placed in a 3L black plastic bucket (1 plant per bucket) with an aerated one-tenth strength modified Hoagland's solution (Shahbaz *et al.,* 2015). Plants were given a 50 nM $CuSO_4$ solution enriched in ^{65}Cu (98% ^{65}Cu and 2% ^{63}Cu) (Isoflex, San Francisco, CA) for 24 h (Pulse24) and 72 h (Pulse72). Growth conditions and treatments are described above.

Chlorophyll PAM fluorescence measurements

Leaf 3 from all treatments were excised between 6-8h in the photoperiod, the petioles placed in DI water, and dark-adapted for 15 min before chlorophyll fluorescence measurements with an FMS system (Hansatech, Norfolk, UK). The program used to estimate chlorophyll fluorescence parameters is detailed in Cohu and Pilon (2007). Actinic light intensities used were 230, 530, and 1250 µmol m^{-2} s^{-1}. The following parameters were measured: photosystem II quantum efficiency (ΦPSII), photochemical quenching (qP), and non-photochemical quenching (NPQ) according to the equations detailed in Murchie and Lawson (2013).

Whole-plant chlorophyll PAM fluorescence imaging

Plants were dark adapted for 15 min before imaging. All work was done in a dark room under dim green light. Briefly, each plant was placed in an Erlenmeyer flask with 10% Hoagland's solution with 98% ^{65}Cu from the individual black buckets in the respective treatment (24 or 72 hours of resupply). Images were taken with a WALZ MAXI Imaging PAM (Effeltrich, Germany). The script contained a saturating pulse intensity of 10 s at a width of 840 ms to measure F_v/F_m followed by measurements of ΦPSII at actinic light intensities of 230, 530, and 1250 µmol m^{-2} s^{-1}. Five to seven Areas of Interest (AOI) were selected for quantitative fluorescence measurements on visible leaves from the top of the canopy in between midribs and major veins (Osório *et al.,* 2014).

Elemental Analysis

Leaves were oven dried at 55°C for 72 hours. Approximately 100 mg of plant material was digested in 1 mL of HNO_3 (70%). The digest was heated for 2 h at 60°C and 6 h at 130°C and subsequently diluted up to 10mL with double distilled water. Samples were analyzed using an ELAN-DRC Inductively Coupled Plasma-Optical Emission Spectroscopy instrument (Pilon-Smits *et al.,* 1999).

Statistical Analysis and Graphics

Statistical analyses were done using R 4.0.3. (R Core Team 2021) and JMP software (version 15.0.0). One-Way ANOVAs were performed using the "lm" function and Tukey mean separation was done using the "emmeans" package (Russell 2021) at a significance level of 0.05 (R Core Team 2021). Two-sample t-tests were performed with the "t.test" function with a significance level of 0.05 (R Core Team 2021). Two-Way ANOVAs were performed using JMP software (version 15.0.0). Principal Component Analyses were completed with JMP software (version 15.0.0). Figures were done using the "ggplott2" package (Wickham 2016).

3.4 RESULTS

Poplar growth and photosynthetic performance

We first aimed to establish that our Cu deficiency treatment produced expected symptoms of deficiency after 6 weeks of growth in hydroponics. The plants grew to the same height until week 4 when Cu deficiency symptoms (stunted growth, leaf curling, chlorosis) typically appear in our hydroponic system (Figure 3.1A). +Cu plants outgrew the Cu deficient plants through week 5 and 6 with significant differences in growth in weeks 4, 5, and 6 (Figure 3.1A). Cu deficiency is known to affect the light reactions of photosynthesis and chlorophyll fluorescence imaging is simple method to evaluate this (Cohu and Pilon 2007, Ravet *et al.,* 2011, Shahbaz *et al.,* 2015). We chose to analyze leaf 3, the first fully developed leaf in order to

compare control and Cu deficient plants. As expected (Shahbaz *et al.,* 2015), there was no significant difference in F_v/F_m (the maximum capacity of PSII) in Leaf 3 in +Cu and -Cu treatments (Table 3.1). ΦPSII was also lower in Cu deficient Leaf 3 compared to +Cu Leaf 3, which indicated that electron transport downstream of PSII was inhibited (Figure 3.1B). Cu deficient Leaf 3 had higher 1-qP values compared to +Cu Leaf 3 which indicated a problem with continual oxidation/reduction of plastoquinone (Figure 3.1C). Non-photochemical quenching (NPQ) was also expectedly lower in Cu deficient Leaf 3 which confirmed -Cu plants displayed a decreased photoprotective capacity (Figure 3.1D). The Cu deficient plants after 6 weeks thus showed typical mild symptoms of deficiency from which plants can normally recover with resupply (Ravet *et al.,* 2011; Shahbaz *et al.,* 2015). These observations were corroborated by measurements of chlorophyll fluorescence in detached leaves (Figure 3.6).

Cu deficiency, mineral distribution, and leaf/stem age

We next investigated how Cu deficiency alters the mineral nutrition of leaves and stems of different age. We defined a developmental gradient of leaf age along the shoot of hybrid poplar by measuring the first seven leaves of +Cu and -Cu plants. Young stems were the stem portion from the apical meristem to Leaf 7 and mature stem was below Leaf 7. Overall, Cu deficiency altered mineral nutrient concentrations in all measured tissue types (Figure 3.2; Figure 3.3). A brief description of the results for each element is given below.

Calcium

Ca was distributed to Leaf 5-7 at a higher concentration versus Leaf 0-2, 3, and 4 in Cu sufficient plants (Figure 3.2A). The same trend was observed in -Cu leaves (Figure 3.2A). Ca levels were higher in -Cu leaves compared to +Cu leaves (Figure 3.2A). Cu and leaf age had a significant effect on Ca distribution in isolation, but not together (Table 3.2). Ca levels in young stems were almost identical between +Cu and -Cu treatments with older stems containing less Ca (Figure 3.2B). Stem age had a small, but significant effect on Ca distribution in young and old stem ($p=0.009$) (Figure 3.2B).

Magnesium

Mg levels were relatively uniform across leaf age in +Cu leaves with Leaf 3 having a slightly higher concentration (Figure 3.2C). A similar trend was observed for Mg in -Cu leaves, but Mg levels were higher in all -Cu leaves (Figure 3.2C). Cu treatment was the only factor with a significant effect on Mg distribution (Table 3.2). There was a ~2000 ppm difference in Mg levels between young stems and mature stems with stem age having a highly significant effect on Mg distribution (Figure 3.2D and Table 3.2). Mg in Cu deficient mature stems was present at very low concentrations (186.7 ppm) and 84% lower than +Cu Mg levels in mature stem (Figure 3.2D).

Phosphorus

Phosphorus was variable across leaf age in +Cu plants with Leaf 3 having the highest amount (Figure 3.2E). Cu deficiency seemed to alter P levels across leaf age with -Cu plants possessing lower levels compared to +Cu plants, although leaf age did not have a significant effect on P levels (Figure 3.2E and Table 3.2). In -Cu stems, there was an ~47% increase in P levels for mature stems and a ~21% increase in young stems (Figure 3.2F). Cu treatment and stem age had both an individual and combined significant effect on P levels in stems (Table 3.2).

Potassium

In the youngest leaves (Leaf 0-2), Potassium levels were almost identical between the treatments (Figure 3.2G). K levels in Leaf 3-Leaf 7 were slightly variable in +Cu plants (Figure 3.2G). In -Cu leaves, K levels decreased steadily with increasing leaf maturity with leaf age have a non-significant effect on K levels (Figure 3.2G). Stems showed similar responses with K levels present a lower concentration in -Cu conditions (Figure 3.2H). Young stems showed a ~60% and old stems showed ~50% reduction in K levels, respectively (Figure 3.2H). Cu treatment and stem age had individual significant effects on K distribution (Table 3.2).

Sulfur

Sulfur levels in Leaf 0-2 were markedly different between +Cu and -Cu treatments (Figure 3.2I). S levels were ~79% lower in -Cu Leaf 0-2 than +Cu Leaf 0-2 (Figure 3.2I). Leaf 3 had the highest S levels in both treatments (Figure 3.2I). We observed a steady decrease from Leaf 4 to Leaf 7 in S levels (Figure 3.2I). Leaf age had a significant effect ($p=0.005$) on S distribution in leaves, but Cu treatment did not (Table 3.2). S levels in young stems were almost identical between treatments (Figure 3.2J). There was ~22% less S in -Cu mature stems compared to +Cu mature stem (Figure 3.2J). There were significant effects on S distribution from stem age and Cu treatment as well as a combined effect (Table 3.2).

Iron

Iron in -Cu plants were higher in all leaves of different age compared to +Cu plants with leaf age having a highly significant effect on Fe distribution (Figure 3.3A). In Cu deficient Leaf 0-2, Fe was ~60% higher and ~76% higher in Leaf 3 versus +Cu leaves (Figure 3.3A). Fe levels in -Cu plants were ~60% higher in Leaves 4-7 compared to +Cu Leaves 4-7 (Figure 3.3A). The same observation was made for young and mature stems (Figure 3.3B). Fe concentration in Cu deficient stems were ~3-fold higher than +Cu stems with Cu treatment having the only significant effect (Figure 3.3B and Table 3.2).

Zinc

Zinc distribution in leaves showed a stair-step pattern with younger leaves having higher Zn in +Cu plants (Figure 3.3C). The same pattern was observed for Zn levels in -Cu leaves, which occur from Leaf 0-2 until Leaf 6 where Zn levels increase (Figure 3.3C). Leaf age and Cu treatment had individual significant effects on Zn levels, but not a combined effect (Table 3.2). Zn was higher in Cu deficient young stems and mature stems with individual and combined significant effect on Zn distribution (Figure 3.3D and Table 3.2).

Manganese

Manganese concentration was similar in Leaf 4-7 of +Cu plants and present at higher levels in Leaf 0-2 and Leaf 3 (Figure 3.3E). In -Cu plants, Mn was lower in Leaf 3 and Leaf 4

while higher in all other leaves (Figure 3.3E). Cu deficient leaves had higher Mn levels compared to +Cu leaves in Leaf 0-2 and Leaf 4-7 with leaf age have a significant effect on Mn levels (Figure 3.3E and Table 3.2). Mn was higher in +Cu stems than -Cu stems with an individual but not combined significant effect on Mn distribution (Figure 3.3F and Table 3.2).

Molybdenum

Molybdenum had a similar trend in leaf age as Zn with older leaves having less Mo in both +Cu and -Cu treatments (Figure 3.3C and 3.3G). Leaf 7 (oldest) was the only leaf age that had higher Mo in the -Cu treatment but only by 13% (Figure 3.3G). Mo was the only element besides Fe to have both an individual and combined significant leaf age effect on element distribution (Table 3.2). Mature stem levels on Mn were similar between +Cu and -Cu conditions (Figure 3.3H). Cu deficient young stems had 85% more Mo compared to +Cu young stems (Figure 3.3H). The only significant effect found in stems was stem age (Table 3.2).

Evaluation of collective mineral distribution in leaves and stems

Principal component analysis (PCA) revealed slightly contrasting influences of tissue age and Cu treatment on overall mineral distribution in leaves versus stems (Figure 3.4). For leaves (Figure 3.4A and 3.4B), the first two principal components, $PC1_{Leaves}$ and $PC2_{Leaves}$, explained approximately two-thirds (47.9% and 18.5%, respectively) of the variation in the macro- and micronutrient concentrations. Leaves from +Cu and -Cu conditions (regardless of age; Table 3.2) separated almost entirely along $PC1_{Leaves}$ (Figure 3.4A), with mostly negative $PC1_{Leaves}$ scores for +Cu conditions and mostly positive $PC1_{Leaves}$ scores for -Cu conditions. Leaves of different age separated along $PC2_{Leaves}$ (Table 3.2), with relatively lower $PC2_{Leaves}$ scores for older leaves and higher $PC2_{Leaves}$ scores for younger leaves (Figure 3.4A). The separation by leaf age along $PC2_{Leaves}$ was more exaggerated in the -Cu conditions compared to the +Cu conditions (Table 3.2), and $PC2_{Leaves}$ scores were generally higher in +Cu versus -Cu conditions (Table 3.2). The minerals underlying these trends formed two distinct clusters: (i) Mg, S, Zn, Mo, and Mn, and (ii) Fe, Ca, K, and P (Figure 3.4B). The second cluster consisted of two

inversely associated pairings that coincided with the separation of +Cu sufficiency (higher K and P) and -Cu deficiency (higher Fe and Ca).

For stems (Figure 3.4C and 3.4D), the first two principal components, $PC1_{Stems}$ and $PC2_{Stems}$, explained over 80% (45.8% and 34.5%, respectively) of the variation in the macro- and micronutrient concentrations. Stems of different ages (regardless of Cu conditions; Table 3.2) separated entirely along $PC1_{Stems}$ (Figure 3.4C), with negative $PC1_{Stems}$ scores for older stems and positive $PC1_{Stems}$ scores for young stems. Stems from +Cu and -Cu conditions separated entirely along $PC2_{Stems}$ (Figure 3.4C), with negative $PC2_{Stems}$ scores for +Cu conditions (similar for mature and young stems) and positive $PC2_{Stems}$ scores for -Cu conditions (slightly higher in mature versus young stems; Table 3.2). $PC1_{Stems}$ was loaded predominantly (79.7%) by S, Mo, Mg, Mn, and Ca, whereas $PC2_{Stems}$ was loaded predominantly (86.6%) by Fe, P, Zn, and K (Figure 3.4D). The latter cluster of minerals consisted of two inversely associated subgroups that coincided with the separation of +Cu conditions (higher K) and -Cu conditions (higher Fe, P, and Zn).

Cu movement in leaf and stem tissues

We developed a method to trace Cu movement through the tissues of hybrid poplar using the stable isotopes of Cu added to the hydroponic growth medium. In order to evaluate the effect of tissue age, we distinguished young leaves that are still expanding rapidly (leaves 0-2) and mature leaves (3, 5, & 7) which were fully developed. There were small but significant increases in both ^{63}Cu and ^{65}Cu in both leaf and stem tissues after 72 h of resupply (Figure 3.5). In leaves and stems, ^{63}Cu and ^{65}Cu were present at the approximate natural abundance in the control treatment (Figure 3.5A-D). Young leaves did not seem to acquire any new ^{65}Cu or ^{63}Cu by 24 hours of resupply (Figure 3.5A). In young leaves, ^{63}Cu increased by ~30% ($p=0.999$) and ^{65}Cu increase ~54% ($p=0.986$) by 72 hours of resupply compared to the -Cu levels (Figure 3.5A). A similar pattern was found in mature leaves for both isotopes. ^{63}Cu increased by ~13% ($p=0.997$) and ^{65}Cu increased ~64% ($p=0.036$) by 72 hours of resupply compared to the -Cu

levels (Figure 3.5B). There were slightly higher increases in both isotopes in young and mature stems (Figure 3.5C and 3.5D). After 72 hours of resupply, ^{63}Cu went up by ~19% in young stems (p=0.995) and ^{65}Cu increased ~79% (p=0.004) (Figure 3.5C). In mature stems, there was no increase in ^{63}Cu (p=0.992) and ^{65}Cu increased ~81% (p=0.001) after 72 hours of resupply compared to -Cu isotope levels (Figure 3.5D). In summary, these observations indicate that some Cu can remobilized from older tissues (increase in ^{63}Cu) and that preferential delivery seems to be to stems versus leaves (increase in ^{65}Cu).

3.5 DISCUSSION

Cu deficiency caused higher mineral concentration in Ca, Mg, S, Fe, Zn, Mn, and Mo in leaves whereas Ca, P, Fe, and Zn in stems were lower under Cu deficiency (Figure 3.2; Figure 3.3). Additionally, there were significant effects of leaf and stem age on mineral distribution for all measured elements, meaning that tissue age seems to be important for some elements (Table 3.2). It was found using stable Cu isotope feeding that some possible remobilization and preferential allocation to stems upon ^{65}Cu enrichment (Figure 3.5).

The maximum capacity of PSII (F_v/F_m) was nearly identical between +Cu and -Cu Leaf 3, which is in agreement with the results of Shahbaz *et al*. (2015). Lower ΦPSII, higher 1-qP, and lower NPQ in Cu deficient plants is expected and with these symptoms plants should still be able to recover. This means that secondary unspecific symptoms (e.g., cell death) are unlikely.

Interestingly, for several elements the change in concentration after Cu deficiency was highly dependent on organ type. For instance, P levels were higher in all leaves of +Cu plants and lower in -Cu plants, but higher in Cu deficient young and mature stems, respectively (Figure 3.2E and 3.2F). In leaves, Cu deficiency caused increases in all micronutrient levels except for Mn levels in Leaf 3 (Figure 3.2E). The largest quantitative differences between +Cu and -Cu treatments for micronutrients can be seen in Fe and Zn levels in leaves and stems (Figure 3.3A-D). This could be due to the cross talk between Fe and Cu homeostasis as observed by Perea-

García and coworkers (2020). These researchers observed an increase in root and shoot Fe concentrations in wild type Arabidopsis under Cu deficiency (Perea-García *et al.,* 2020). Additionally, it has been observed that Cu deficiency induces the expression of Cu-II chelate reductase activity in root tips (Bernal *et al.,* 2012). In Arabidopsis, these reductases are encoded by ferric reductase/oxidase 4/5 which are conserved in plants (Bernal *et al.,* 2012).

Furthermore, in our data, Mo levels decreased as leaves became older and were higher in the Cu deficient treatment (Figure 3.2G). Crosstalk between Cu and Mo homeostasis is not unexpected, given that Cu is required for the synthesis of molybdenum co-factor (Kuper *et al.,* 2004). Mo also followed the same distribution trend as S in leaves (Figure 3.2I; Figure 3.3G). Mo is taken up as molybdate, an oxyanion that is taken up by members of the sulfate transporter family (Tomatsu *et al.,* 2007). Billard and colleagues (2014) studied the effects of Cu deficiency on Cu remobilization, Mo accumulation, and chloroplast protein changes in *Brassica napus*. It was observed that Cu deficiency had no effect on N, Ca, K, S, P, B, Fe, Mn and Zn uptake (Billard *et al.,* 2014). However, the authors did observe that Mo uptake increased by 121% and the increased expression of a Mo uptake gene (*MOT1*) occurred under Cu deficiency (Billard *et al.,* 2014). We see the opposite effect in hybrid poplar where in most cases, leaf age and Cu status had a significant effect on mineral distribution except for Mg, P, and K (Table 3.2).

Siebrecht and colleagues (2003) examined the diurnal variations in nutrient concentrations in the xylem sap of hydroponically grown poplar. These investigators observed that Mg, Ca, K, NO_3^-, $H_2PO_4^-$, and SO_4^{2-} reached their maximum concentrations in the first half of a 16 h photoperiod while photosynthesis and transpiration (mL H_2O h^{-1} g^{-1} FW leaf) remained constant (Siebrecht *et al.,* 2003). This study also examined leaf age finding that K levels remained constant from young leaves to old leaves (37 leaf plants), Mg and Ca levels were higher in older leaves, and S plateaued at Leaf 10 of 37 (Siebrecht *et al.,* 2003). These results

indicated that leaf age, along with photosynthesis and transpiration can determine nutrient distribution along the shoot of poplar.

Remobilization of nutrients is another strategy used by plants when nutrient deficiency or leaf senescence occurs (Maillard *et al.,* 2015). Maillard *et al*. (2015) examined remobilization of 13 nutrients during nutrient deficiency and leaf senescence in cultivated crop leaves and woody species. The authors found a low net remobilization efficiency for field grown black poplar (*Populus nigra*) for all measured nutrients compared to English oak (*Quercus robur*) (Maillard *et al.,* 2015). Under Cu deficiency, *Brassica napus* was found to have a low remobilization score (%) as well (Maillard *et al.,* 2015). It could be that the 3 weeks of Cu deficiency increases the onset of leaf senescence, which could trigger early signals for nutrient remobilization (Maillard *et al.,* 2015). Furthermore, stable isotopes can be used to trace nutrient remobilization in plant tissues. Benatti and colleagues (2014) used the stable isotopes of Cu (^{63}Cu and ^{65}Cu) to discover that metallothionein proteins are needed to remobilize Cu from sensing *Arabidopsis* leaves.

Our data revealed significant interactions between Cu status, tissue type, and tissue age (Figure 3.2; Figure 3.3; Table 3.2). The principal component analysis results show clear distinctions that suggest that mineral distributions in leaves are influenced heavily by Cu deficiency and slightly by leaf age (Figure 3.4A). Surprisingly, Fe did not cluster with the same group as Mo, Mn, and Zn whose levels where higher under Cu deficiency (Figure 3.4B; Figure 3.3A-G) (Carrió-Seguí *et al.,* 2019; Bernal *et al.,* 2012). However, K and P did cluster together with the same trend in leaves (higher concentration in +Cu conditions) (Figure 3.4B; Figure 3.2A; 3.2E; 3.2G). The separation of stems along $PC1_{Stems}$ and $PC2_{Stems}$ revealed a clustering that suggests developmental stage has a strong influence on mineral distribution and, to a lesser degree, Cu status (Figure 3.4C). For Cu, it has been suggested before that delivery is prioritized to leaves for use in photosynthesis after deficiency, but for stems it has been unclear (Shahbaz *et al.,* 2015). Interestingly, our data now show that most of the ^{65}Cu fed to deficient

plants was allocated to stems in the first 3 days of resupply (Figure 3.5). It would be worth investigating how the mineral compositions of leaves and stems changes once Cu is introduced back into the hydroponic system.

The increases in ^{63}Cu and ^{65}Cu observed in the leaves from the pulse experiment were very small compared to the stems (Figure 3.5). The increase in ^{63}Cu by day 3 in young leaves could represent remobilization from older tissue via the phloem (Figure 3.5A). There was a larger increase in ^{65}Cu in mature leaves and stems compared to young leaves and this could indicate the ^{65}Cu detected in the stems was still in route to target tissues (e.g., leaves) for cofactor assimilation (Figure 3.5B-D). The results from this experiment possibly indicated slight remobilization to younger tissues. It could also represent a poplar specific phenotype consistent with observations by Maillard *et al.* (2015) that *P. nigra* was classified as a low net remobilization species for macro and micronutrients. However, *P. nigra* tissue in this study was harvested from wild species along the edges of grassland or from pot experiments in the greenhouse. The age of the *P. nigra* trees were estimated to be several decades old compared to our poplar saplings grown in hydroponic culture (Maillard *et al.,* 2015). It was unclear what specific role leaf age plays in mineral distribution under Cu deficient conditions.

3.6 CONCLUSIONS

In conclusion, Cu deficiency revealed quantitative changes in leaf and stem mineral concentration and possibly alteration in uptake strategies due to Cu deficiency. Specifically, Cu deficiency caused higher mineral concentration in Ca, Mg, S, Fe, Zn, Mn, and Mo in leaves and Ca, P, Fe, and Zn in stems. Principal Component Analysis revealed a clear influence of organ age on mineral distribution in leaves and stems with clear clustering of young and mature organs. PCA also revealed distinct clusters of elements whose concentrations were significantly altered by Cu deficiency (Mn, Mg, S, Mo, and Zn). Stable isotope data revealed some Cu remobilization and preferential allocation to the stem of poplar after ^{65}Cu enrichment.

3.7 FIGURES AND TABLES

Table 3.1. F_v/F_m of +Cu and -Cu Leaf 3. Values represented as mean ± 1SE. +Cu (n=6) -Cu (n=6).

Treatment	F_v/F_m
+Cu	0.83 ± 002
-Cu	0.83 ± 004

Table 3.2. Results of Two-way ANOVAs for the effects of +Cu/-Cu conditions and leaf/stem age on mineral distribution in hybrid white poplar grown in 10% Hoagland's. The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (*n.s.* = not significant).

Mineral(s)	Leaves			Stems		
	Age	Cu	Age × Cu	Age	Cu	Age × Cu
Ca	***	***	*n.s.*	*	*n.s.*	*n.s.*
Mg	*n.s.*	***	*n.s.*	***	**	***
P	*n.s.*	***	*n.s.*	***	**	***
K	*n.s.*	***	***	***	***	*n.s.*
S	**	***	*n.s.*	***	*	*
Fe	***	***	*	*n.s.*	***	*n.s.*
Zn	**	***	*n.s.*	**	***	*
Mn	*	**	*n.s.*	**	*	*n.s.*
Mo	***	***	*	***	*n.s.*	*n.s.*
$PC1_{Leaves}/PC1_{Stems}$	*n.s.*	***	*n.s.*	***	*n.s.*	*n.s.*
$PC2_{Leaves}/PC2_{Stems}$	***	***	***	*	***	*

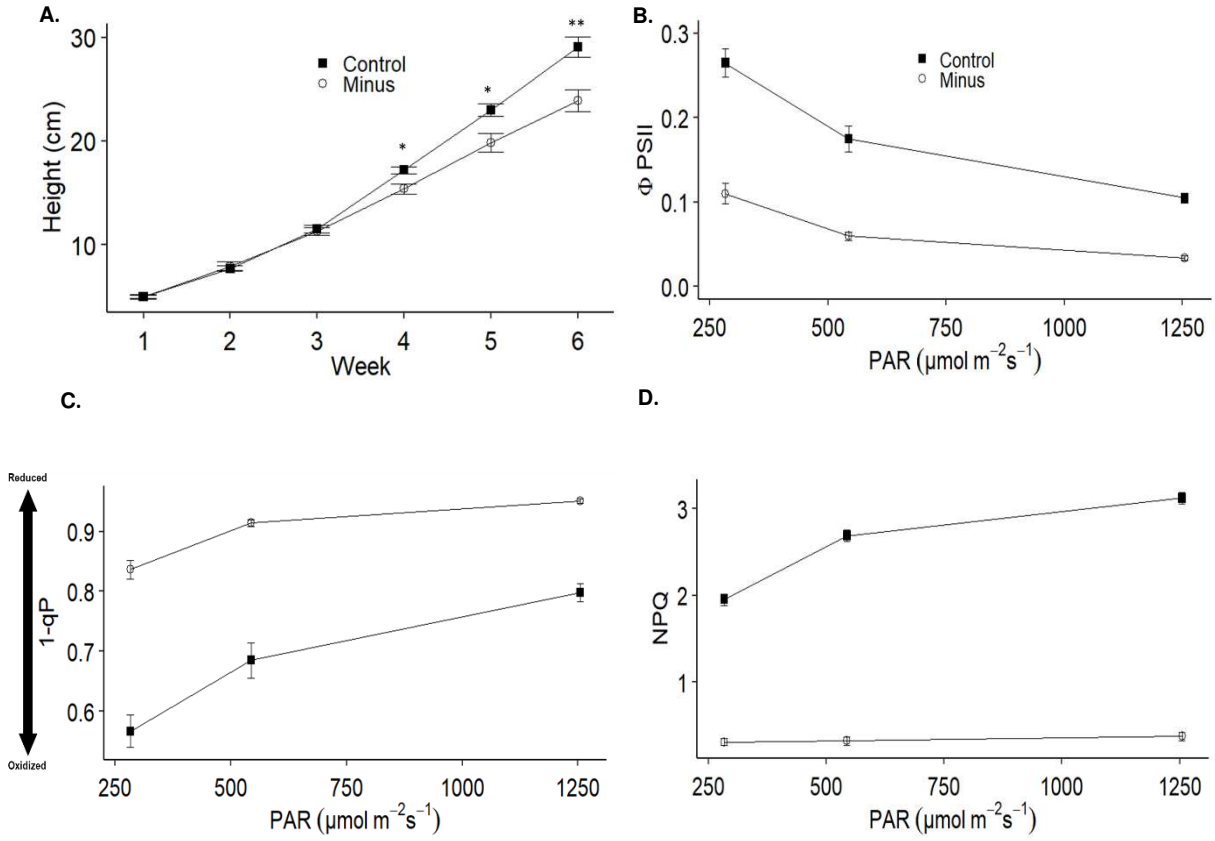

Figure 3.1. Effect of Cu deficiency on plant growth and photosynthesis in Leaf 3 from 6 weeks in hydroponics. A. Plant growth of +Cu and -Cu plants. +Cu (n=6). –Cu (n=6). Mean ± 1SE. **B.** The quantum efficiency of PSII (ΦPSII) in Leaf 3 as a function of light intensity (Photosynthetically Active Radiation or PAR). **C.** 1 – qP, representing the redox state of the plastoquinone pool as a function of PAR for Leaf 3. **D.** Non-Photochemical Quenching (NPQ) for Leaf 3 as a function of PAR. ΦPSII, 1-qP, and NPQ were analyzed using an FMS system. *Closed squares*: control + Cu, *open circles:* – Cu plants, Values are represented as mean ± 1SE. +Cu (n=5) and -Cu (n=6). The asterisks denote significant differences; * = $P < 0.05$, ** = $P < 0.01$.

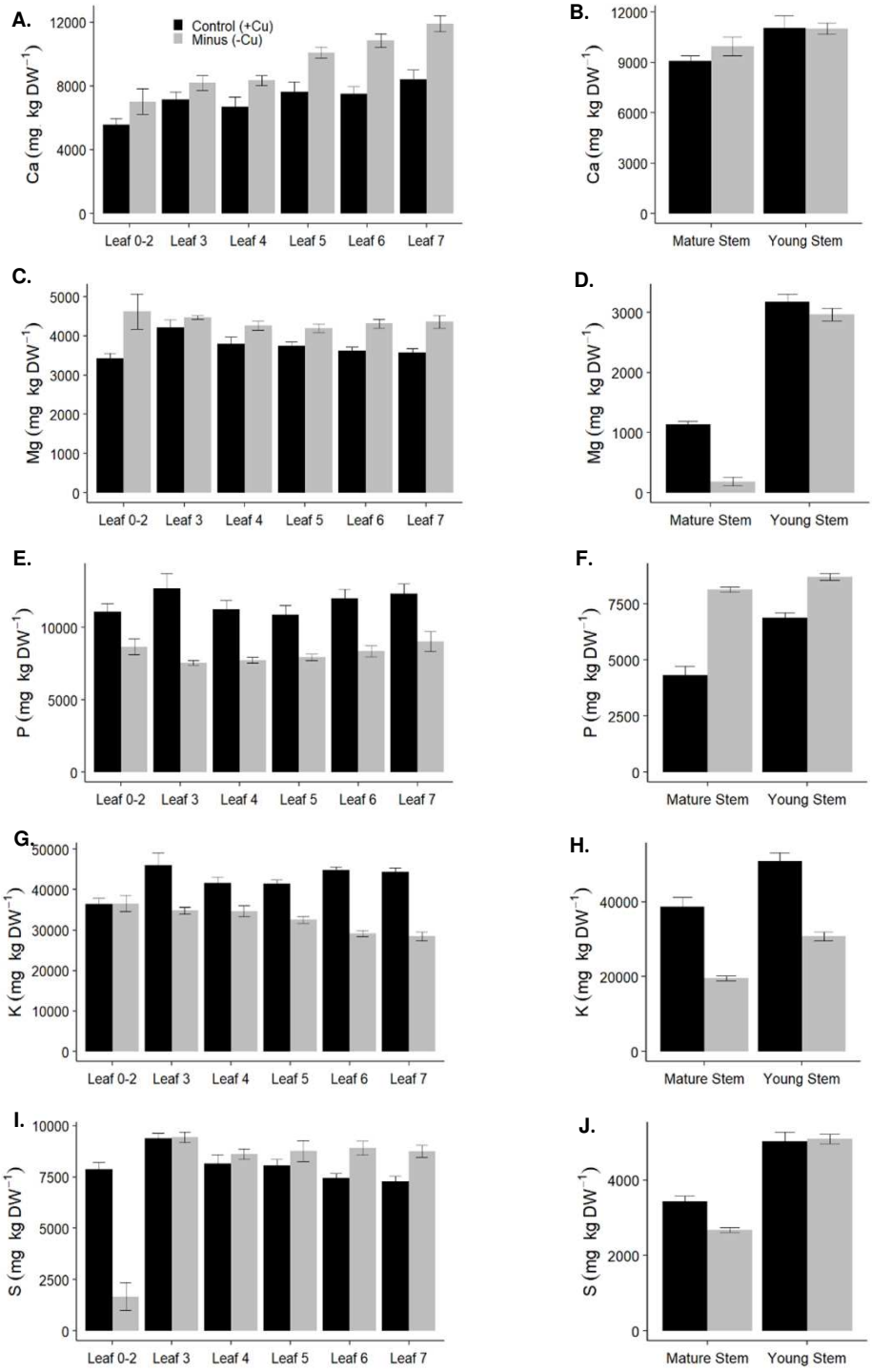

Figure 3.2. Distribution of macronutrients as a function of leaf and stem age and Cu feeding status. Values represented as mean ± 1 SE. +Cu and -Cu (n=6).

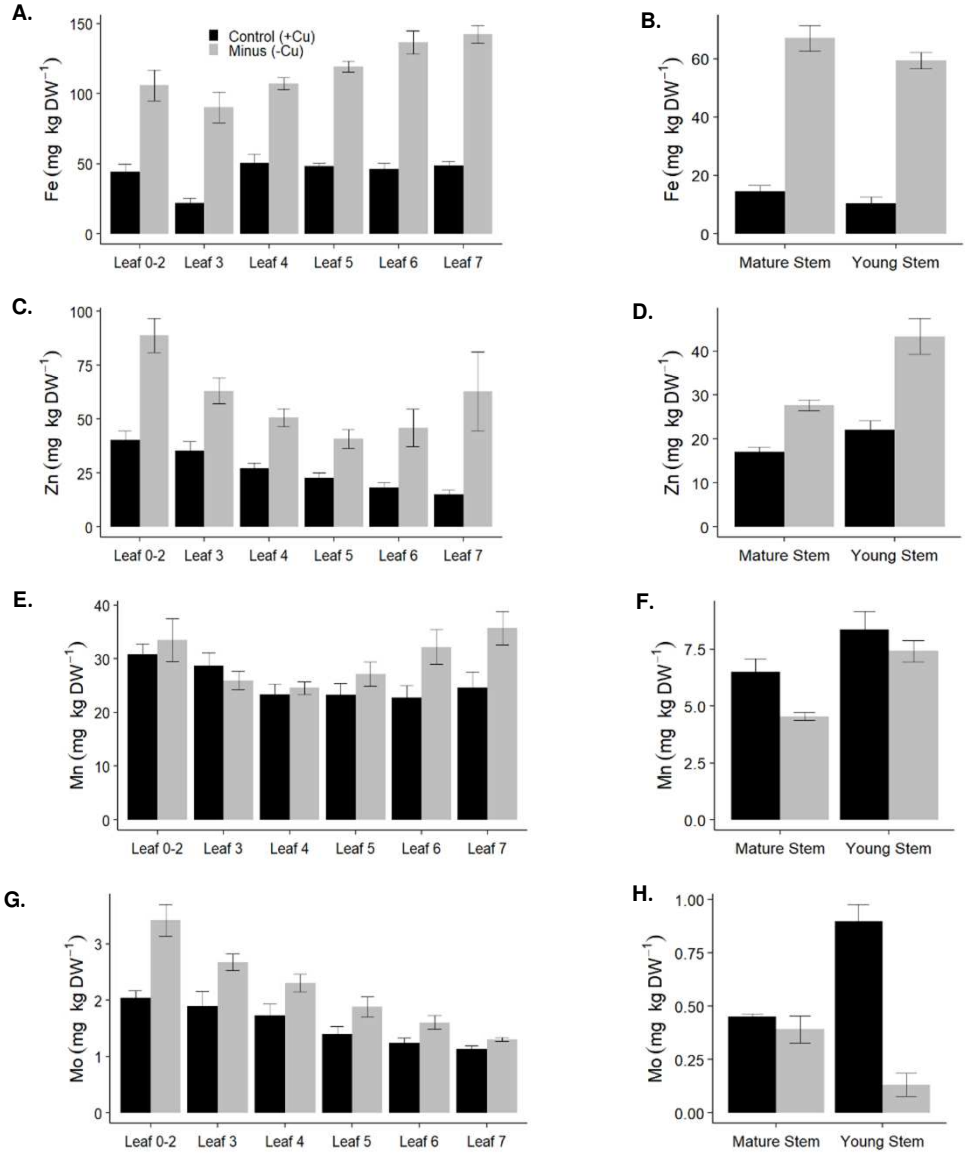

Figure 3.3. Distribution of micronutrients as a function of leaf and stem age and Cu feeding status. Values represented as mean ± 1 SE. +Cu and -Cu (n=6).

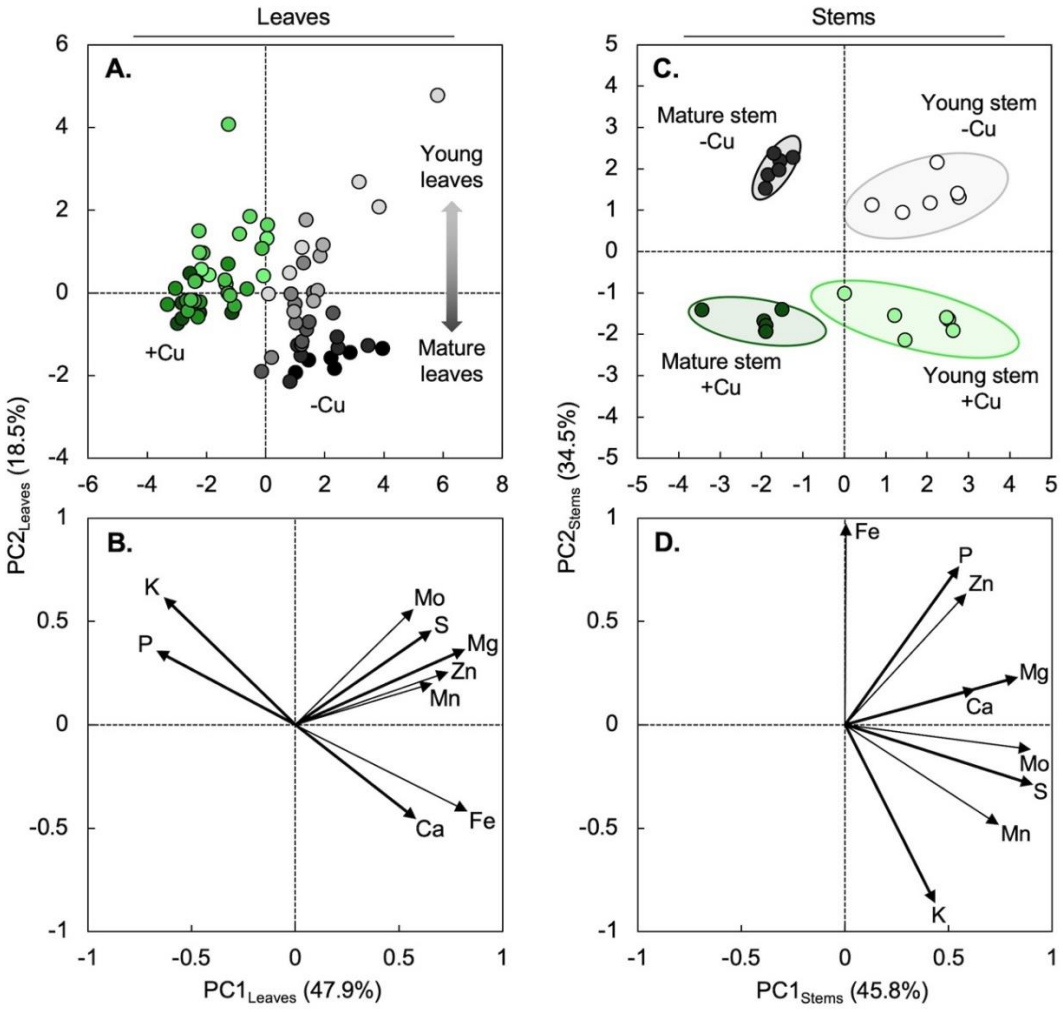

Figure 3.4. Principal component analysis (PCA) of mineral distribution in leaves and stems from poplar grown in +Cu and -Cu conditions. A. Score plot of the first two principal components ($PC1_{Leaves}$ and $PC2_{Leaves}$) for leaves. Leaves from +Cu conditions are represented by a green color gradient (light: young, dark: mature). (n=6). Leaves from -Cu conditions are represented by a grayscale gradient (light: young, dark: mature). (n=6). **B.** Loading plot of macro- and micronutrients on $PC1_{Leaves}$ and $PC2_{Leaves}$. **C.** Score plot of the first two principal components ($PC1_{Stems}$ and $PC2_{Stems}$) for stems. Density ellipses (p=0.95) for each group are labeled accordingly. (n=6). **D.** Loading plot of macro- and micronutrients on $PC1_{Stems}$ and $PC2_{Stems}$.

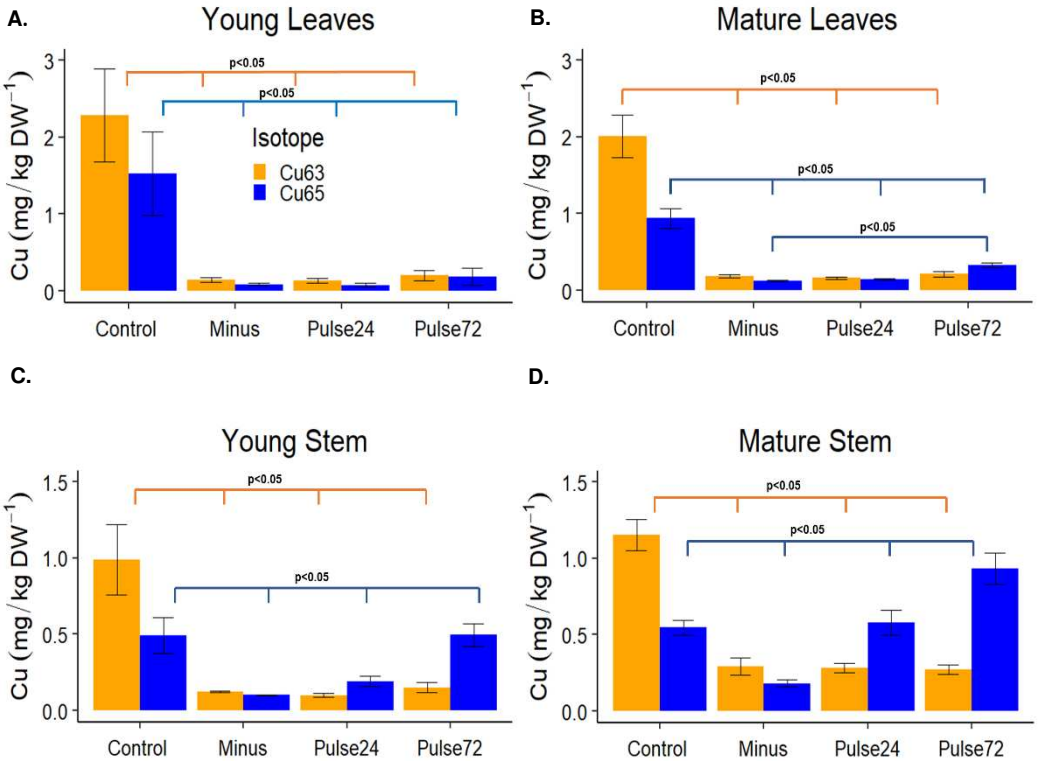

Figure 3.5. Cu movement in leaves and stems. A. Isotopic concentration in young leaves. +Cu (n=3), -Cu, Pulse24, Pluse72 (n=4). **B.** Isotopic concentration in mature leaves. +Cu (n=3), -Cu, Pulse24, Pluse72 (n=12). **C.** Isotopic concentration in young stem. +Cu (n=3), -Cu, Pulse24, Pluse72 (n=4). **D.** Isotopic concentration in mature stem. +Cu (n=3), -Cu, Pulse24, Pluse72 (n=4). Values are shown as mean ± 1SE. One-way ANOVA results with significance are indicated with bars corresponding to isotope colors.

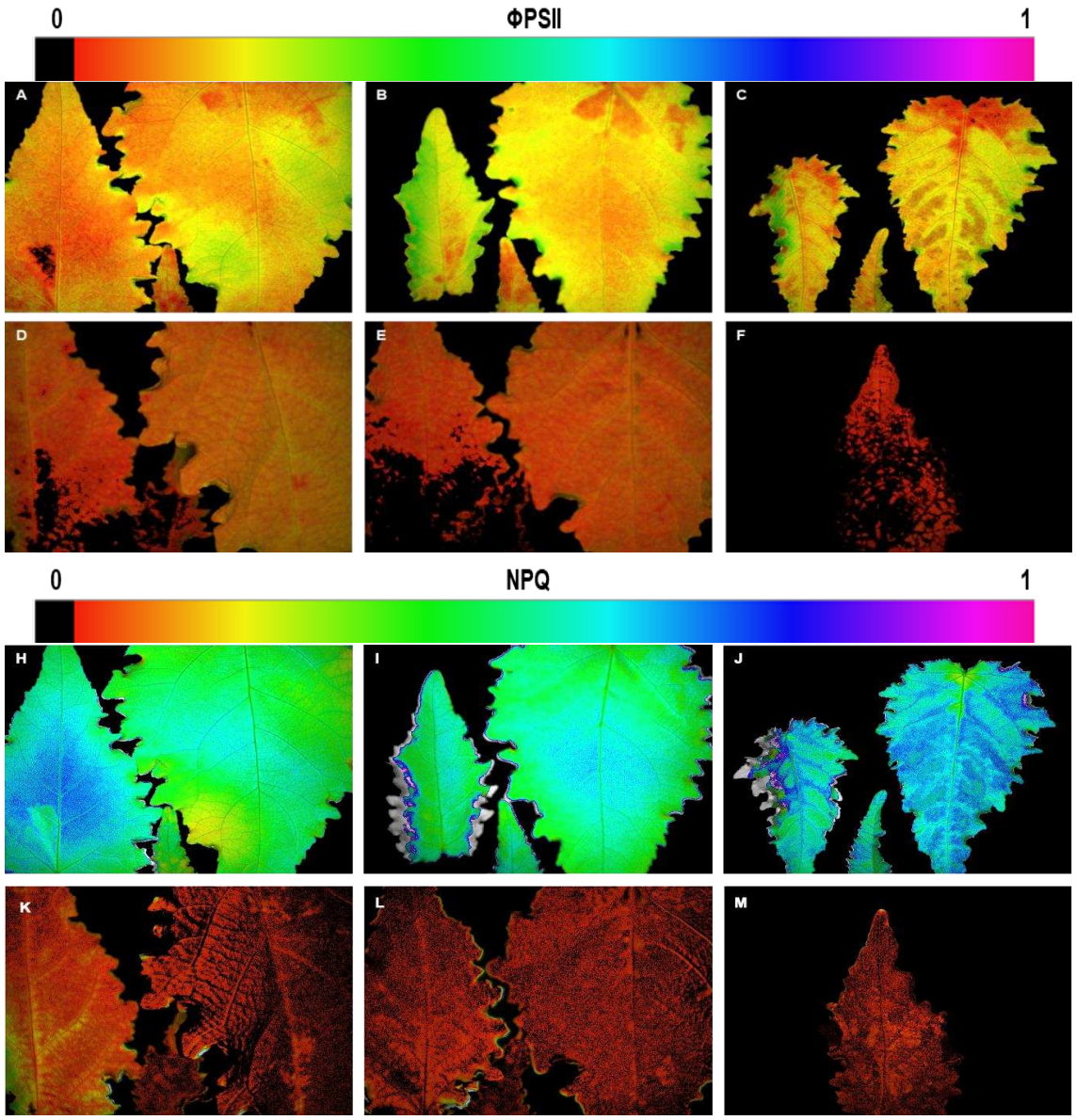

Figure 3.6. ΦPSII and NPQ in Leaf 3 (middle), 5 (left), and 7(right) from +Cu and -Cu plants grown in 10% Hoagland's with and without 50 nM $CuSO_4$. A-C.+Cu leaves (n=3) **D-F.** -Cu leaves and (n=3). PAR: 230 µmol m^{-2} s^{-1}. Scale represents values from 0-1 for each parameter. Data shown captured with WALZ® Imaging PAM.

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CHAPTER 4: THE EFFECTS OF COPPER DEFICIENCY ON LIGIFICATION, XYLEM VESSEL STRUCTURE, AND HYDRAULIC TRAITS IN HYBRID POPLAR[3]

4.1 SUMMARY

Copper (Cu) homeostasis is integrated with many plant physiological processes including lignification of plant cell walls. This link occurs through Cu's role as a cofactor in the apoplastic laccase enzymes that oxidize monolignols that form the hydrophobic lignin polymer, which provides rigidity and strength to the water transport system. In this study, we investigated the effect of Cu deficiency on lignin content and chemistry in poplar stems. We also examined the effect of Cu deficiency on the stiffness of stem wood and leaf cell walls. Cu deficiency resulted in significant reduction in lignin content and a shift in the guaiacyl (G) to syringyl (S) monomer ratio (S/G) of stem xylem. Accompanying these stem traits, Cu deficient stems were also more elastic (i.e., lower modulus of elasticity) than +Cu stems. In contrast with these results, Cu deficient leaves had markedly higher modulus of elasticity, pointing to stiffer mesophyll cell walls. These results suggest that a lack of Cu can cause structural defects in leaf cell walls and alter the lignin polymer composition of stems in poplar.

4.2 INTRODUCTION

Copper (Cu) is a cofactor in many plant proteins that are involved in biochemical redox reactions including lignin polymerization, photosynthesis, respiration, and reactive oxygen species metabolism (Printz *et al.,* 2016). In cell wall metabolism, Cu is utilized in the laccase enzymes which are encoded by *LAC* genes to oxidize monolignols in the apoplast just before lignin polymerization via radical coupling (Berthet *et al.,* 2012; Wang *et al.,* 2013). Lignin is an

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important polymer in plant cell walls that provides biomechanical support and plant pathogen defense (Vanholme *et al.,* 2010; Bhuyian *et al.,* 2009). It was recently discovered that the Cu-containing uclacyanin (UCC) proteins UCC1 and UCC2 are required for lignification of a nanodomain in the Casparian strip in *Arabidopsis* (Reyt *et al.,* 2020). The *ucc1/ucc2* double mutant in this study displayed a disruption in mineral nutrient homeostasis and an increased permeability of endodermal cells when compared to the wild type (Reyt *et al.,* 2020). Moreover, Hoffman and coworkers (2020) observed the co-localization of laccases and iron (Fe) containing peroxidases in the lignified secondary cell walls of *Arabidopsis* stems. Specifically, AtLAC4, AtLAC17, and AtPRX72 localized in secondary cell walls of xylem (Hoffman *et al.,* 2020). It has been observed at the whole-plant level that Cu deficiency can cause reduced lignification and altered stem structure in conifer tree species ranging from *Pseudotsuga menziesii* to *Pinus radiata* (Oldenkamp *et al.,* 1966; Ruiter 1969). The irreversible bending and twisting of stems are an observed phenotype of Cu deficient conifers (Turvey and Grant 1992). In hybrid poplar, Cu deficiency in young leaves is associated with stunted plant growth (Shahbaz et al. 2015). Despite these observations, direct connections between Cu homeostasis, lignification, water transport, and cell wall chemistry and structure in trees is part of a biological process with many unanswered questions.

Cu homeostasis and lignification have been mainly investigated through examination of Cu deficiency and toxicity symptoms. For example, increased Cu supply has been shown to increase lignin content in soybean roots within 24 to 72 hours (Lin *et al.,* 2005). In *Brassica juncea*, it was shown that exposure to Cu oxide nanoparticles caused an increase in the lignification of hypocotyls based on phloroglucinol-HCl staining, with a particular increase in xylem vessel lignification (Nair *et al.,* 2015). Interestingly, *LAC* gene expression has also been shown to respond to Cu status in hybrid poplar (Shahbaz *et al.,* 2015). The mRNA levels of *LAC12* recovered with resupply in young leaves while *LAC40* expression was higher under Cu deficiency.

The chemical composition of poplar wood consists of 50% cellulose, 30% hemicellulose, and 20% lignin (Balatinecz *et al.,* 2001). The chemical structure and composition of these three components affect the structural and physiological functioning of wood (e.g., elasticity and strength of vascular and ground tissues). Of particular interest are the highly lignified conduits responsible for water transport in flowering plants – xylem vessels. Considering that maximal rates of photosynthesis and growth are closely aligned with xylem water transport (Boyce *et al.,* 2009; Brodribb & Field 2000), and that xylem vessels are subjected to large negative pressures (ca. < -1 MPa) during normal operation, it is critical that vessels be both highly conductive and resistant to crushing forces (Hacke *et al.,* 2001; Jacobsen *et al.,* 2007).

Lignins are structural cell wall polymers that provide rigidity and strength for xylem vessels. It has been observed that reduction in lignin can affect vessel shape and function in poplar (Voelker *et al.,* 2011). Voelker and coworkers (2011) used transgenic poplar with down-regulated *4-coumarate:coenzymeA ligase* (4-CL) to study the hydraulic architecture and xylem structural integrity in low-lignin trees. Microscopic evidence from control trees showed regularly shaped and lignified xylem vessels throughout the growth ring, whereas low-lignin trees possessed deformed vessels near the pith (Voelker *et al.,* 2011). As for water transport traits, it was found that low-lignin poplars were more vulnerable to xylem embolism (the filling of vessels with gas) and had lower survival compared to control trees (Voelker *et al.,* 2011). Given that hydraulic conductance scales to the 4^{th} power of vessel diameter (Scholz et al. 2013), vessel diameter is another important xylem trait that affects water transport (Hacke *et al.,* 2017). Poplar vessel diameters vary and can be influenced by their location (e.g., roots, stem, branch, leaf), growth environment, and genotype (Hacke and Sauter 1996; Enquist *et al.,* 1999; Olson *et al.,* 2013). Vessel diameters in stems reported by Voelker et al (2011) from control and low lignin transgenic hybrid poplar ranged from 30-45 µm. Typical values of xylem-specific conductivity (flow rate normalized by pressure gradient, length, and cross-sectional area; K_s) of Poplar stems range from about 5-8 kg m^{-1} s^{-1} MPa^{-1} (Plavcová and Hacke 2012).

Lignins consist of cross-linked *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units which are all derived from phenylalanine in the phenylpropanoid pathway (Mentz *et al.,* 2018). A crucial component of lignification is the monomer ratio typically referred to as the syringyl:guaiacyl or the S/G ratio (Vanholme *et al.,* 2010). The S/G ratio informs the degree of polymerization of the lignin polymer (Studer *et al.,* 2011). However, the precise physiological function of the typical S/G ratio in woody angiosperms is not fully understood. Studer and coworkers (2011) found the S/G ratio ranged between 1.0 and 3.0 among 1,100 *Populus* species. Lima et al (2018) investigated the link between total lignin, lignin composition, and xylem embolism resistance in dryland forest species. These investigators found that leaf lifespan was strongly correlated with xylem embolism resistance (Ψ_{50}) and the S/G ratio but found that total lignin was not correlated with either leaf lifespan or xylem embolism resistance (Lima *et al.,* 2018). Thus, the authors suggested that increased leaf lifespan and lower xylem embolism vulnerability in this species resulted from a wider S/G ratio, rather than higher total lignin (Lima *et al.,* 2018). Aligned with this idea, it has been proposed that G-rich lignin is more hydrophobic and rigid in nature (more cross-linking) when compared to S-rich lignin (Pereira *et al.,* 2017). Greater cell wall rigidity may therefore arise, at least in part, from the more frequent cross-linking of G-rich lignin (Koehler and Telewski 2006). Taken together, these data suggest that alteration of the S/G ratio can have a direct impact on water transport and the biomechanical properties of wood.

In this study, we investigated how Cu deficiency alters the lignin content and its intersection with xylem structure, hydraulic traits, and carbohydrate composition in hybrid poplar. We hypothesized that Cu deficiency would lower the lignin content in stems and decrease the stiffness of mesophyll cell walls in the leaf. We also hypothesized that Cu deficiency would result in reduced vessel diameter and xylem-specific conductivity because of reduced xylem lignification. Following from these hypotheses, we set out to answer the following questions: (1) Can we detect structural and hydraulic differences in Cu deficient shoots? (2)

Does Cu deficiency reduce the lignin content in hybrid poplar? (3) Are the lignin monomer and structural carbohydrate composition altered by Cu deficiency? (4) Does Cu deficiency lead to a decrease in vessel diameter and xylem-specific conductivity?

4.3 METHODS

Plant material and growth conditions

Hybrid white poplar (*P. tremula* x *P. Alba*, INRA 717-1B4) seedlings were propagated *in vitro* on ½ strength Murashige and Skoog (Sigma-Aldrich) hormone free media with sucrose (20 g/L) and agar (7 g/L). For hydroponic growth, 3-month-old seedlings were removed from the agar and the roots were washed with DI water. Explants that were rooted and at least ~8 cm tall, were randomly distributed to 20-L black plastic buckets with an aerated one-tenth strength modified Hoagland's solution (3 plants per bucket) (Hoagland and Arnon, 1950; Shahbaz *et al.,* 2015). The hydroponic solution pH was adjusted to 5.9 with KOH (Shahbaz *et al.,* 2015). Seedlings were immediately covered with Magenta® boxes to maintain high humidity. The covers were gradually lifted and removed over the first 7 days. Plants were grown in a climate-controlled room under a light intensity of 150 µmol m^{-2} s^{-1}, with a 16h day and 8h night photoperiod. Temperature was maintained at 22°C±1°C. Cu sufficient treatments ("+Cu") were given a 50 nM $CuSO_4$ solution. Cu deficient treatments ("-Cu") had $CuSO_4$ ommitted from the nutrient solution.

Chlorophyll PAM fluorescence measurements

Leaf 3 from all treatments were excised after between 6-8h in the photoperiod. The petioles were immediately placed in DI water and the leaves were dark-adapted for 15 min before measuring chlorophyll fluorescence using an FMS system equipped with a leaf clamp (Hansatech, Norfolk, UK). Measurements were made in the center of one half of the leaf. The program used to estimate chlorophyll fluorescence parameters is detailed in Cohu and Pilon (2007). Actinic light intensities used were 230, 530, and 1250 µmol m^{-2} s^{-1}. The following

parameters were measured as in Murchie and Lawson (2013): photosystem II quantum efficiency (ΦPSII), photochemical quenching (qP), and non-photochemical quenching (NPQ).

Pressure volume curve (PV) experiments

Fully expanded leaves were harvested randomly at 6 weeks from each treatment between 6-8h in the photoperiod. Leaves were excised at the base of the petiole with a razor blade. Each leaf was weighed to the nearest 0.0001g and the leaf water potential was measured with a Scholander Pressure bomb (Model 3005, Soil Moisture Equipment Corp). Leaf relative water content (RWC) was calculated as: (fresh mass − oven-dry mass)/(initial fully turgid mass − oven-dry mass). The bulk modulus of elasticity (ε) was calculated as the change in leaf water potential divided by the change in RWC at turgor loss (Gleason *et al.,* 2021). The water potential at the turgor loss point was objectively extracted from each curve using a custom written R-script (Gleason *et al.,* 2021).

Sample preparation, TBO staining, light microscopy, and hydrualic traits

Stems were stored in 70% EtOH at 4°C until processing. Stem cross sections were prepared and stained according to Mitra *et al* (2014) with some modification. Briefly, each stem was hand-sectioned at the same distance from the top of the plant with a fresh razor blade (Olson *et al.,* 2014). Each cross section was soaked in DI H_2O for ~1 min and transferred to 0.02% Toluidine Blue stain. Cross sections were imaged with a Lecia® CTR5000 microscope equipped with a color camera. Images were captured with Lecia Application Software and processed with ImageJ software (https://imagej.nih.gov/ij/). D_h (hydrualically weighted diameter) and k_s (theoreitcal xylem-specific conductivity) were calculated according to Lewis and Boose (1995).

Stem deflection mesurements.

The lower 5 cm of 20 cm stem long segments of comparable thickness (~ 3 mm) were horizontally clamped. At 15 cm from the fixation point a string was attached to which increasing weight was applied by filling a plastic container with water. The amount of weight was recorded

that was required for each extra cm deflection as measured with a ruler up to 10 cm. Finally the weight was recorded at which stems broke.

Lignin quantification, S/G monomer composition, and strucutral carbohydrate analysis

Oven-dried plant stems from +Cu and -Cu treatments were ground with a Wiley Mill to pass through a 40-mesh screen and then soxhlet extracted for 24 hours (Coleman *et al.,* 2008). A modified Klason method was used to quantify lignin content according to Coleman et al (2008) with 3 mL of 72% H_2SO_4. Lignin monomer ratios were quantified using the thioacidolysis procedure from Robinson and Mansfield (2009). Structural carbohydrate conentrations were determined using high-performance liquid chromatography (HPLC) according to Robinson and Mansfield (2009).

Statistical Analysis and Graphics

Analyses and figures were done using R 4.0.3. Figures were done using the "ggplott2" package for R (Wickham 2016). Two-sample t-tests were performed with the "t.test" function with a significance level of 0.05 (R Core Team 2021).

4.4 RESULTS

Growth, photosynthetic performance, and Cu and Fe levels in poplar

After six weeks of growth, the height of plants in the +Cu treatment exceeded the height of Cu deficient plants (Figure 4.1A). Cu deficiency in leaves resulted in decreased photosynthetic electron transport (Shahbaz *et al.,* 2015). As expected, the Cu deficient plants had lower ΦPSII at all three measured actinic light intensities (283, 544, and 1255 μmol m^{-2} s^{-1}) in Leaf 3 from the Cu deficient treatment (Figure 4.1B). Cu levels in young leaves (Leaf 0-2) taken from plants in the Cu+ treatment were ~3 ppm whereas Cu was below the limit of detection in the -Cu leaves (Figure 4.1C). Fe levels were 3-fold higher in the deficient treatment compared to +Cu leaves (Figure 4.1D).

Poplar stem deflection and strength under Cu deficiency

Cu deficiency has been reported to cause stem weakness in trees. To compare the mechanical strength of the stems of Cu deficient poplar plants with plants grown on +Cu media we measured bending for horizontally clamped sections of stem as a function of applied mass at 15 cm from the point where the stems were fixed. We found that stems of Cu deficient plants bent significantly more per unit mass (i.e., force) applied and break at a much lower applied mass, indicating a strongly reduced mechanical strength (Figure 4.2).

Pressure volume curves

We investigated the effect of Cu deficiency on cell wall elasticity in poplar leaves using the pressure volume curve technique (Schulte & Hinckley 1985). Three parameters were calculated from the PV curves: the modulus of cell wall elasticity (ε), Relative Water Content (RWC) at the turgor loss point, and the water potential at the turgor loss point (Ψ_w). We were especially interested in ε given the direct role of Cu in lignin polymerization via the laccase enzymes and the effect of Cu deficiency on cell wall structure and chemistry (Lu *et al.,* 2013). There was a small but significant difference ($p= 0.0133$) in Ψ_w (turgor loss point) between +Cu (0.77 ± 03 MPa) and -Cu leaves (0.72 ± 09 MPa) (Table 1). For RWC at the turgor loss point, there was a small but non-significant ($p=0.669$) difference between +Cu (0.96) and -Cu leaves (0.98) (Table 4.1). Cu deficient leaves had a 78% higher ε ($p=0.006$) than +Cu leaves (22.4 ± 4.0 MPa vs 102.7 ± 21.2 MPa) (Table 4.1).

Hydraulic traits in poplar stems

To investigate the impact of Cu deficiency on water transport traits, we used light microscopy images to calculate three hydraulic traits: the hydraulically weighted diameter (D_h), theoretical xylem-specific hydraulic conductivity (K_s), and vessel density. D_h and K_S were statistically similar between the Cu treatments, although both tended to be slightly higher in the +Cu treatment (Table 4.2). Vessel density on the other hand was slightly higher in the -Cu treatment, although this result was also non-significant ($p=0.154$) (Table 4.2).

TBO histochemical staining of +Cu and -Cu stems

Next, we used histochemical staining and light microscopy to qualitatively assess the lignification of the xylem in stems from both treatments. We observed that lignification of the younger stems did not differ between the control and treatment plants (Figure 4.3A and 4.3B; Figure 4.5). The younger tissue appeared to have similar patterns of lignification as well as similar numbers of xylem vessels (Figure 4.3A and 4.3B; Figure 4.5). In contrast with this, cross sections taken from older parts of the stem revealed a distinct difference between the +Cu and -Cu stems (Figure 4.3C and 4.3D; Figure 4.5). +Cu stems from mature parts of the stem had a wider xylem growth ring while -Cu xylem growth rings appeared narrower with fewer numbers of total vessels (Figure 4.3C and 4.3D; Figure 4.5). Many of the vessels in the Cu deficient plants also appeared to have thicker secondary cell walls (Figure 4.3D; Figure 4.5).

Quantification of lignin and structural carbohydrates from +Cu and -Cu poplar stems

We answered the question whether or not Cu deficiency would result in lower lignin content, altered lignin monomer ratio (S/G), or altered structural carbohydrate composition/concentration. Cu deficient stems had a 4.6% lower amount of Klason lignin versus +Cu stems ($p<0.001$) while acid soluble lignin was 1.7% higher in the -Cu stems ($p<0.001$) (Figure 4.4A). Furthermore, Cu deficiency significantly shifted the S/G ratio towards higher S lignin content compared to control stems ($p<0.001$) (Figure 4.4B). The overall composition of the structural carbohydrates was very similar between +Cu and -Cu stems, with glucose being the most abundant at 518.5 µg/mg in +Cu stems and 503.8 µg/mg in -Cu stems (Figure 4.4C). However, there were significant differences between arabinose, galactose, glucose, mannose, and xylose between the treatments (Figure 4.4C).

4.5 DISCUSSION

Our data revealed a direct and significant reduction in Klason lignin under Cu deficiency, but an increase in acid-soluble lignin (Figure 4.4A). This is complementary to our observations of TBO stained tissue (Figure 4.3C and 4.3D). Interestingly, the S/G ratio also increased under

Cu deficiency, meaning there were more S units in -Cu stems (Figure 4.4B). The lignin polymer that contains more S units is hypothesized to be more linear in nature and possesses less cross linking (Dumitrache *et al.,* 2016). Assuming there is a reduction in laccase expression (Shahbaz *et al.,* 2015), and thus enzyme function (e.g., less lignin polymerization in the cell wall), this could explain the shift in the S/G ratio. The S/G ratio shift and its relationship to the structural carbohydrates is consistent with findings by Dumitrache and co-workers (2016), who observed little to no change in structural carbohydrate concentration with differing S/G ratios. The physiological and ecological significance of an optimal S/G ratio is still unclear, but our results show that Cu status can have a significant effect on the chemistry and structure of the lignin polymer.

The PV curve experiment revealed that the Cu deficient leaves were stiffer than the +Cu leaves, contrary to what was hypothesized. This could be related to lignin content or compostion in the leaf similar to what was reported by Lima et al (2018). It could be that G rich lignin content was higher in these leaves causing higher ridity and resulting in stiffer cell walls (Pereira *et al.,* 2017; Koehler and Telewski 2006). Our results seem to be different from Robson (1981) who described an increase in alpha-cellulose and reduction in lignin in Cu deficient wheat leaves, which may have indicated a defect in leaf mesophyll cell walls.

As for functional hydraulic traits, the difference between D_h and K_s was non-significant, but was lower for both traits under Cu deficiency compared to control stems (Table 4.2). The values of Dh and K_S were consistent with what has been reported by Plavcová and Hacke (2012). Vessel diameter is critical component for the calculation of both D_h and K_s and the vessel diameters were similar between +Cu and -Cu treatments. Given that there were more vessels per unit cross section in the -Cu treatment, vessel number cannot explain the lower K_s in -Cu stem cross sections. However, we note that vessel density, Dh, and K_S were not staistically significant between the Cu treatments, and it remains a possibility that Cu defficiency does not affect the density or size of xylem vessels. It may also be possible that lower lignin content might affect

the thickness of the secondary cell walls of xylem vessels, thus decreasing their crushing resistance (Hacke *et al.,* 2001). Nair et al. (2015) noted when *Brassica juncea* was exposed to Cu toxicity the lignification of the xlyem vessels increased. However, we did not observe any differences in vessel wall thickness between +Cu and -Cu stem cross sections.

4.6 CONCLUSION

Our data show the alteration of the lignin composition induced by Cu deficiency for the first time in hybrid poplar. We found that Cu deficiency had an adverse impact on the lignification and stiffness of poplar stems, but did not have a significant effect on the hydraulic capacity of these stems, nor the density and size of xylem vessles. Interestingly, we observed the opposite results of our hypothesis that -Cu conditions would result in more flexible leaf cell walls. Rather, we observed a marked increase in cell wall stiffness in Cu deficient leaves. Taken together, our results suggest that the elasticity of wood and leaf tissues is markedly affected by Cu deficiency. However, it remains an important research question why the elasticity of wood and leaf tissues responded in opposite directions to Cu deficiency. We recommend that further study into this question should include the quantification of cellulose, hemicellulose, and lignin fractions of leaves, stems, and root tissues.

4.7 FIGURES AND TABLES

Table 4.1. Pressure volume curve parameters of +Cu and -Cu poplar leaves. +Cu (n=6) and -Cu (n=8). *MPa:* Megapascal. *RWC*: Relative Water Content. Ψ is the water potential at the turgor los point. The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$ (t-test).

Treatment	Modulus of elasticity (ε) (MPa)	RWC at Turgor Loss Point (%)	Ψ at Turgor Loss Point (MPa)
+Cu	22.4 ± 4.0**	0.96	0.77 ± 03*
-Cu	102.7 ± 21.2**	0.98	0.72 ± 09*

Table 4.2. Hydraulic traits from +Cu and -Cu poplar grown in 10% Hoagland's solution. +Cu (n=3). -Cu (n=3). D_h is the hydraulically weighted diameter in µm. k_s is the therotical xylem-specific conductivity. Values represented as Mean ± 1SE.

Treatment	D_h (µm)	Theoretical K_s (kg m^{-1} s^{-1} MPa^{-1})	Vessel density (vessels mm^{-2})
+Cu	33.7 ± 2.9	6.1 ± 1.3	195.0 ± 25.5
-Cu	29.3 ± 1.4	4.7 ± 1.0	249.5 ± 13.4

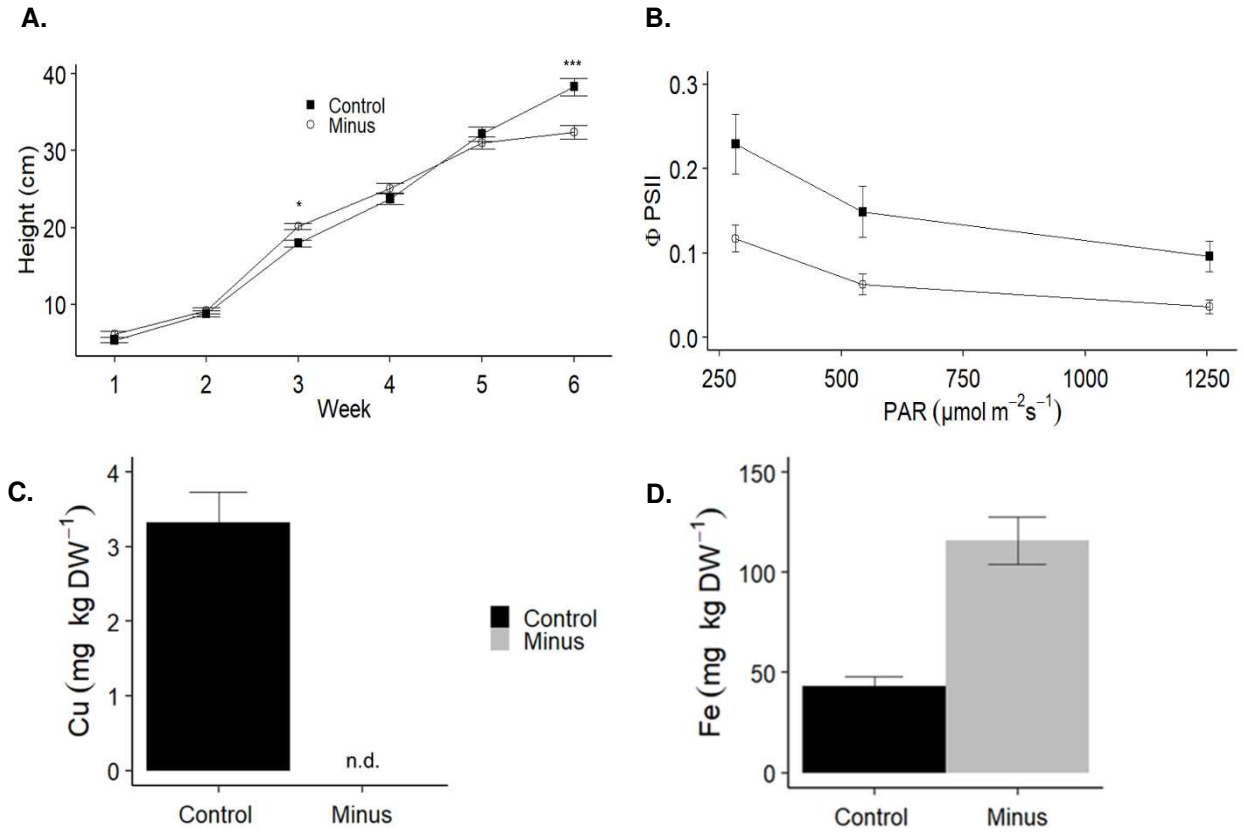

Figure 4.1. Growth, photosynthetic performance and Cu and Fe levels in leaves of +Cu and –Cu hybrid poplar. Plants were grown in 10% Hoagland's solution for 6 weeks. **A.** Plant growth of +Cu and -Cu plants. +Cu (n=11). –Cu (n=11). Mean ± 1SE. **B.** The quantum efficiency of PSII (ΦPSII) in Leaf 3 as a function of light intensity (Photosynthetically Active Radiation; PAR). *Closed squares*: control +Cu, *open circles:* –Cu plants. **C.** Cu levels in Leaf 0-2. **D.** Fe levels in Leaf 0-2. Values are expressed as Mean ± 1SE. Control (n=6) Minus (n=5). The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

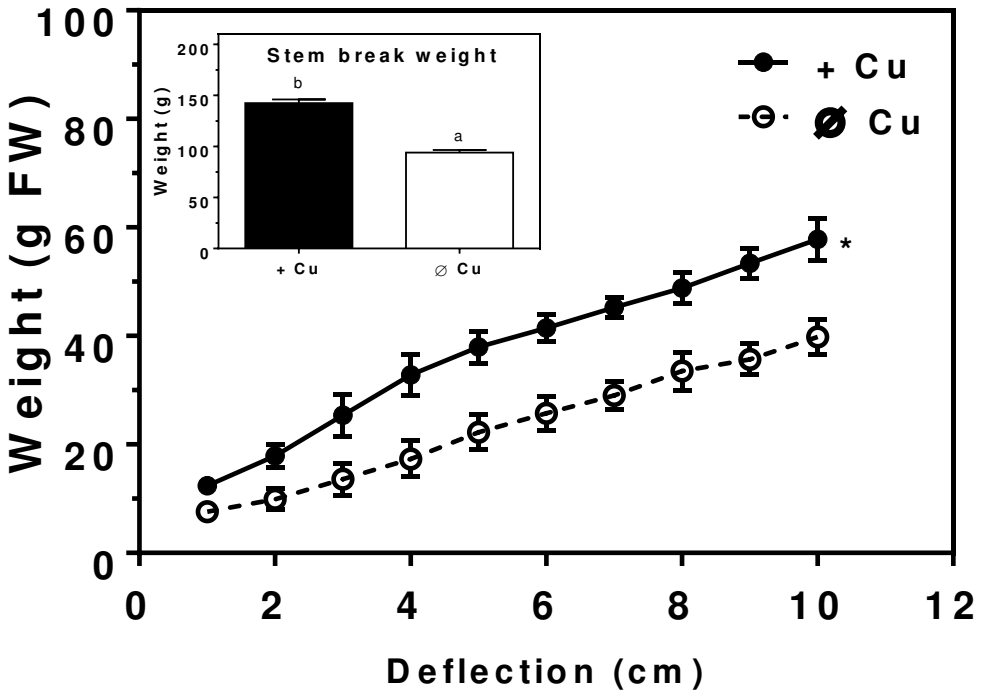

Figure 4.2. Effect of Cu deficiency on stem deflection and stem strength of hybrid poplar grown in +Cu and -Cu conditions. Stem deflection for the first 10 cm was measured by increasing mass at the end of stem section, finally the weight required for the breaking point was recorded (bar graph). Values are given as averages ± SD (n = 3). The asterisks denote significant effects; * = $P < 0.05$ (t-test).

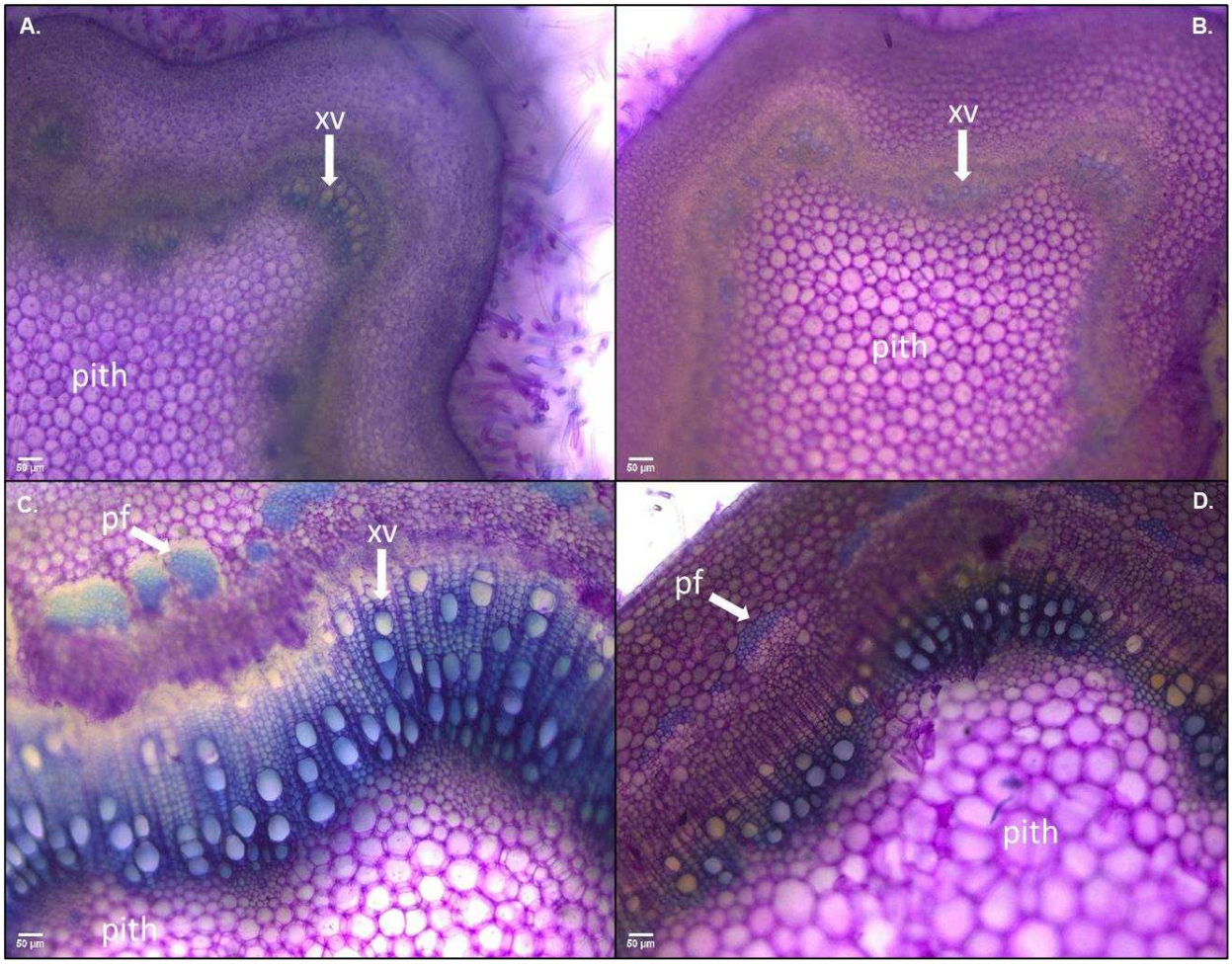

Figure 4.3. TBO staining of stem cross sections of +Cu & -Cu poplar. Stems were hand sectioned from young (top) stems and older (middle) stems. **A.** Young stem from a +Cu plant (100X) **B.** Young stem from a -Cu plant (100X) **C.** Mature stem from a +Cu plant (100X) **D.** Mature stem from a -Cu plant (100X). xv: xylem vessel, p: pith, pf: phloem fiber cells. Images representative of 3 biological replicates.

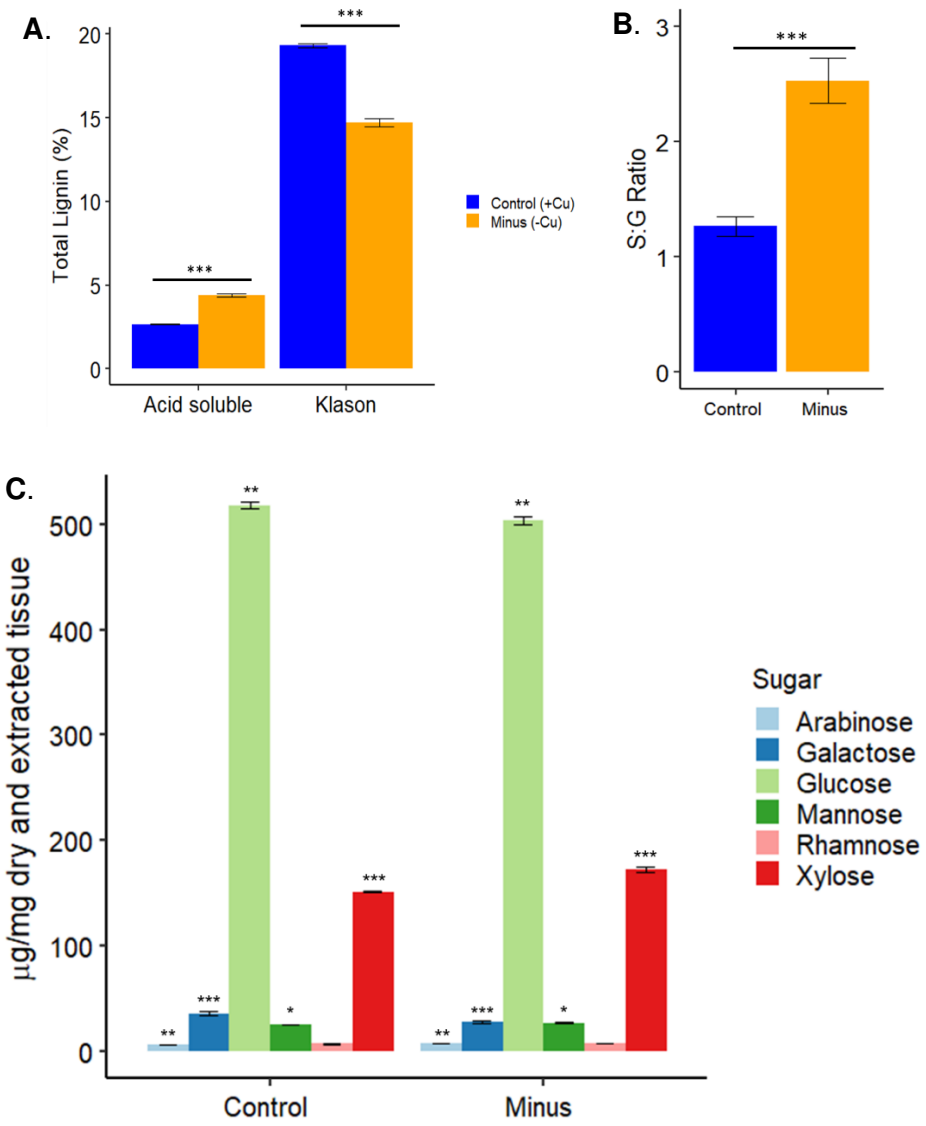

Figure 4.4. Total lignin, changes in lignin monomer compostion, and structural carbohydratres in +Cu and -Cu poplar stems. A. Acid soluble and Klason lignin from +Cu and -Cu stems. (n=11). **B.** Cu deficiceny inudced changes in syringyl and guaiacyl lignin monomer compostion. (n=11). **C.** Structural carbohydrate concentrations in +Cu and -Cu stems. (n=11). The asterisks denote significant effects; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (t-test).

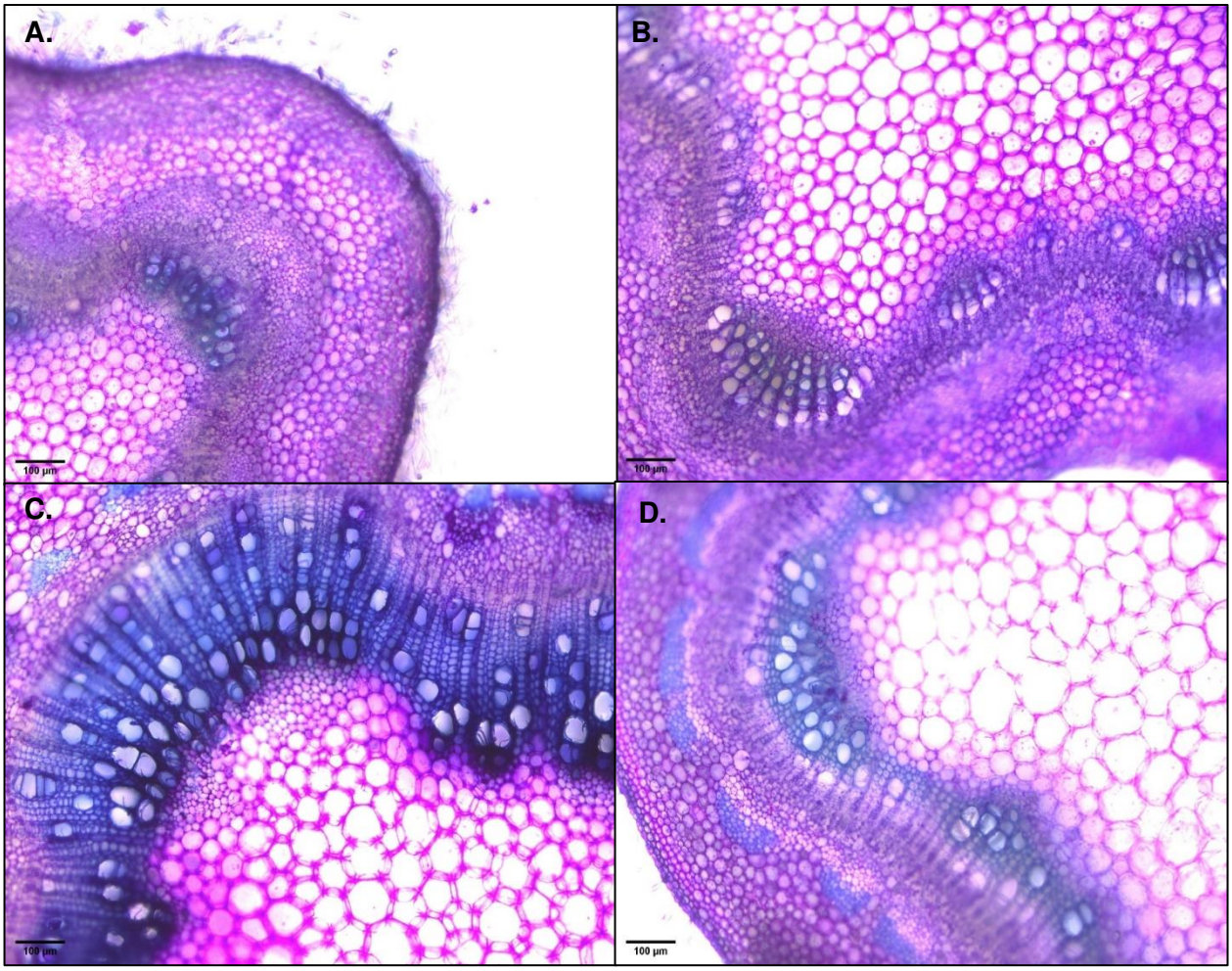

Figure 4.5. TBO stain of +Cu and –Cu poplar stems grown in 10% Hoagland's solution. Each stem section was hand sectioned wit a razor blade and stained with TBO. **A.** +Cu young stem cross section. **B.** –Cu young stem cross section **C.** +Cu older stem. **D.** –Cu older stem.

4.8 LITERATURE CITED

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CHAPTER 5: SUMMARIZING DISCUSSION

Copper metabolism in plants is integrated into many physiological processes including photosynthesis and lignification of plant cell walls. In order for photosynthesis to properly function, Cu must be delivered to target tissues via plant conduits (xylem and phloem) for cofactor assimilation into Cu-proteins (Burkhead *et al.,* 2009). In the case of Cu deficiency, plants can experience structural and chemical alterations that have direct impact on growth, photosynthesis, development, and water transport. With Cu homeostasis being a nexus of plant physiological function, it is critical to understand how processes at the leaf and whole plant level (e.g., photosynthesis and stomatal conductance) work in tandem to drive its homeostasis.

Systemic Cu transport and distribution in leaves and stem and prioritization to photosynthesis

The first goal of this dissertation was to examine priorities for Cu transport at the leaf and whole plant levels after deficiency. More specifically, I aimed to investigate the physiological factors that take part in driving this prioritization. In Chapter 2, I first developed a method to trace Cu movement through poplar that was grown in a hydroponic system. The method used stable isotopes of Cu (^{63}Cu and ^{65}Cu) which can be traced separately by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) to track long distance transport and provide a quantitative approach to deciphering how Cu is partitioned and prioritized in poplar. This project was a follow up to the work of CSU post-doctoral researcher Dr. Muhammad Shahbaz who characterized the Cu deficiency response in hybrid white poplar and conducted the initial Cu resupply experiments (Shahbaz *et al.,* 2015). Using Inductively Coupled Plasma-Mass Spectrometry, we detected Cu isotopes in leaves and stem. We found that the 98% ^{65}Cu we supplied plants with was the major of the two isotopes detected in the young leaves and mature leaves after 72h of resupply. We also used chlorophyll fluorescence to measure changes in photosynthesis upon Cu resupply. This revealed a rapid recovery of photosynthetic electron transport in a young leaf. The chapter

2 data showed phenotypic recovery from Cu deficiency symptoms in leaves at 24h and 72h of ^{65}Cu enrichment. In addition to the method developed to trace Cu movement, we examined isotope enrichment coupled with chlorophyll fluorescence imaging in young and mature leaves as a proxy to visualize photosynthetic recovery because of Cu delivery from the leaf vasculature to photosynthetic electron carriers. We also measured stomatal conductance (g_s) in the first six weeks of growth on young and mature leaves and in the 3-day ^{65}Cu enrichment period. The lamina between the primary and secondary veins exhibited rapid and complete recovery of ΦPSII upon pulse with ^{65}Cu. During deficiency, mature leaves maintained a higher g_s than younger leaves, but three days after Cu resupply the younger leaves that had recovered showed the highest g_s. The results presented in chapter 2 indicate that Cu delivery to photosynthesis is prioritized by leaf age with young leaves being given priority. Data on stomatal conductance hints at fast recovery of xylem-mediated Cu transport to younger leaves during Cu resupply. Finally, the data in chapter 2 provide a quantitative understanding of how Cu is systemically transported and distributed to photosynthetic and stem tissues in hybrid poplar saplings.

Organ age as driver of altered mineral distribution induced by Cu deficiency

Nutrient deficiency has been known to cause metabolic remodeling at several levels of organization in plants (Marschner 2012; Billard *et al.,* 2014; Shahbaz *et al.,* 2015). In the case of Cu deficiency, the Cu-miRNAs are upregulated so that available Cu can be prioritized for use in photosynthetic electron transport (Ravet *et al.,* 2011; Shahbaz *et al.,* 2015; Shahbaz and Pilon, 2019). In Chapter 3, we examined the effect of Cu deficiency on the altered mineral distribution in leaves and stems of poplar as well as Cu movement in these tissues. We found element-specific trends and significant interactions between Cu status, tissue type, and tissue age that were induced by Cu feeding status. It was observed that Cu deficiency caused higher mineral concentrations of Ca, Mg, S, Fe, Zn, Mn, and Mo in leaves and Ca, P, Fe, and Zn in stems.

Principal Component Analysis, a statistical technique that finds patterns in multidimensional data sets, revealed distinct clusters that suggested that developmental stage played a role in the altered distribution of essential nutrients. We also observed preferential allocation to the stem when plants were pulsed with 98% ^{65}Cu. These results indicated that developmental stage of an organ as well as Cu feeding status can drive nutrient distribution and allocation.

Cu deficiency, lignification, and water transport traits in poplar

There are reports dating back to the 1960's which suggested that Cu deficiency had negative effects on tree growth (Oldenkamp *et al.,* 1966; Ruiter 1969). Irreversible bending and twisting of stems had also been noted as a phenotype of Cu deficiency in conifers (Turvey and Grant 1992). Since these observations, research on roles of Cu in stems and vasculature has focused on the Cu-containing laccases that polymerize monolignols that form the heteropolymer lignin that provides protection and rigidity in xylem vessel cell walls (Vanholme *et al.,* 2010; Bhuyian *et al.,* 2009: Zhao *et al.,* 2013; Lu *et al.,* 2013; Hoffman *et al.,* 2020). This has raised questions regarding how Cu deficiency alters the structure, chemistry, and water transport traits that contribute to physiological functioning of poplar.

In Chapter 4, we investigate the changes in chemistry and structure of poplar xylem under Cu deficiency. Since xylem vessel structure directly affects how efficiently water is transported and lignin plays a crucial structural role in xylem cell walls, we were interested in how Cu deficiency directly impacts these structures. Moreover, we also measured the theoretical xylem specific conductivity (k_s) and hydraulically weighted diameter (D_h) that inform on water transport through the xylem. We used a Scholander pressure bomb to build pressure-volume (PV) curves for fully expanded leaves from +Cu and -Cu hydroponic conditions. We found that the water potential and relative water content at the turgor loss point were very similar. However, the modulus of elasticity, which indicates how flexible the cell wall is was much higher in the -Cu leaves. This result was contrary to our hypothesis based on what is

known about cell wall chemistry and the reported biomechanics of lignin (Koehler and Telewski 2006). The reduction of Cu in leaves was expected to reduce the stiffness of the mesophyll cell walls possibly due to a reduction in lignin. Our findings suggest that the cell walls in the leaf were much stiffer compared to the +Cu leaves giving insight into structural and chemical changes possibly induced by Cu deficiency. We also used lignin staining and light microscopy to assess lignin differences and quantify vessel diameter and theoretical water conductance through poplar xylem vessels. The analysis of lignin revealed a narrower growth ring and the unaffected water transport capacity of the xylem vessels under Cu deficiency. This suggests that Cu deficiency can directly alter the lignin structure while water transport is seemingly unaltered, and vessels are still operational.

In the work completed in this dissertation, Cu homeostasis was investigated on a new level. Plant organs and tissues are physically connected to support critical plant physiological processes such as, photosynthesis and water transport. The work in this dissertation addresses how plant mineral nutrition plays a key role in both processes and how they are integrated. First, the stable isotope work from Chapters 2 and 3 indicate that new Cu can be prioritized to stem and leaf tissue after deficiency. It also indicated that traits such as stomatal conductance, which helps regulate water delivery to photosynthesis can respond to Cu supply. Another novel conclusion that arose from this research is that Cu deficiency can affect the homeostasis of other minerals in a new and unexpected way. Cu thus affects several other elements rather than just Fe, S and Mo (Carrió-Seguí *et al.,* 2019; Bernal *et al.,* 2012). This points to another affected level imposed by Cu deficiency other than just leaf biochemistry of gene expression, protein accumulation and Cu enzyme function. It also demonstrates that organ type (e.g., leaf and stem) and tissue age, coupled with Cu status can affect essential nutrient allocation in the plant. Furthermore, we show that Cu deficiency affects the structure of the stem at the level of the lignin polymer and demonstrate that Cu is necessary for proper lignification. Most importantly, the stem is needed for proper and optimal water delivery for leaf photosynthesis and the xylem

is the major highway for this process to function (Boyce *et al.,* 2009; Brodribb & Field 2000). Lignin provides the strength and rigidity needed in the stem xylem to withstand the large negative pressures (ca. < -1 MPa) during normal operation. It also provides the stiffness needed to with stand wind, gravity, snow, and other physical forces (Hacke *et al.,* 2001). These structures are also required for the recovery of aerial tissues from deficiency, which we demonstrated in Chapter 2.

Outlook and future research directions

While our research sheds light on quantification of stable Cu isotopes at the organ level, future focus will need to be on transport in plant conduits (xylem and phloem). In trees, our work, and the work of Cao et al (2020) are the first two reports aimed at deciphering Cu transport with ours being focused on utilization in photosynthesis (Hunter *et al.,* unpublished). However, important questions remain. How is Cu systemically transported and distributed? What traits are direct drivers of long distance Cu transport? It would be interesting to repeat the pulse experiment with 98% ^{65}Cu in the Cu sufficient treatment and perform Laser Ablation-ICP-MS on leaves of different age to gain better understating of native Cu transport in poplar (Wu *et al.,* 2009). This technique would not only quantify the isotopes, but it would provide an image that shows the distribution of Cu across the leaf. Additionally, using a LI-COR600® which would capture stomatal conductance and chlorophyll fluorescence data at the same time would be exciting. This would give photosynthesis and g_s data in real-time compared to our destructive sampling methods. It may also be interesting to measure hydraulically weighted diameter, xylem and leaf specific conductivity (D_h, k_s, and k_{leaf}) on a Cu sufficient plant grown in 98% ^{65}Cu. This would show where Cu is moving in the plant in addition to the hydraulic traits in the leaf and stem that are contributing to its movement.

There are many environmental and physiological factors that drive essential nutrient distribution: photosynthesis, transpiration rates, organ size, developmental stage, nutrient

bioavailability, gene expression, etc. While our work quantified the distribution of macro and micronutrients under Cu deficiency, much work is needed to understand additional environmental and physiological factors that explain these developmentally driven observations. By which mechanisms can developmental stage have a significant effect on essential nutrient distribution? One approach to answer this question could be to perform a physiological trait network analysis as completed by Gleason et al (2021). In this approach, traits are measured and analyzed as a network (conceptual and quantitative) rather than in isolation (Gleason *et al.,* 2021). Bivariate correlation could be used to assess if there are significant linear relationships between elemental concentration, transpiration rate, and photosynthesis across the different leaf ages, followed by the production of a conceptual trait network.

Evidence regarding plant nutrition, water transport, and cell wall chemistry have only been partially connected. It is assumed that nutrients are carried through the xylem via the transpiration stream to respective sites of utilization. What is the exact link between Cu, lignin chemistry, and water transport? It would be interesting to know if there is any lateral distribution of Cu along the transpiration stream through the xylem, for use in lignification. This would tell us if there were any competition for Cu delivery between photosynthesis and lignification. It could be that our results from Chapter 3 are showing this via preferential allocation to the stem. Scanning electron microscopy (SEM) is often used to examine the ultrastructure of the xylem (pit membranes) (Jansen *et al.,* 2007). It is also coupled to energy dispersive X-ray spectroscopy (EDX) to analyze the surface of materials and can measure element distribution and concentration. SEM-EDX could be used to measure Cu concentration in a stem section. It might be a useful what to see how Cu is spatially distributed along the xylem vessels in stems.

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