Differences in aluminum tolerance among *Populus genotypes* –

Development of methodology for identification of poplar genotypes suitable for acidic forest land.

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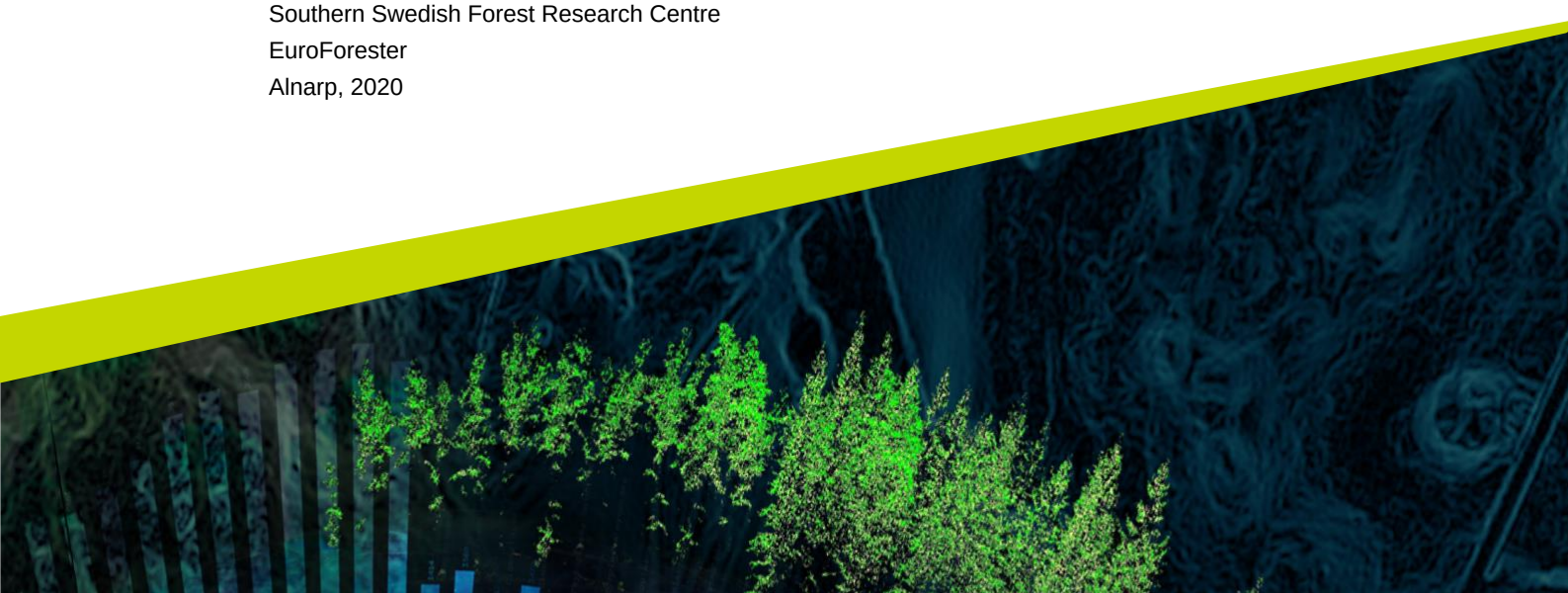
Master Thesis • (30 hp)
Swedish University of Agricultural Sciences, SLU
Southern Swedish Forest Research Centre
EuroForester
Alnarp, 2020



Difference in aluminum tolerance among *Populus* genotypes – Development of methodology for identification of poplar genotypes suitable for acidic forest land

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Credits:	**(30 hp)**
Level:	**(Second Cycle, A2E)**
Course title:	**Masters Thesis in Forest Science**
Course code:	**EX0984**
Programme/education:	**Euroforester MSc**
Course coordinating dept:	**Southern Swedish Forest Research Centre**
Place of publication:	**Alnarp**
Year of publication:	**2020**

Keywords: *Populus*; Aluminum; hematoxylin staining; clonal variation; soil pH; short-rotation forestry;

Swedish University of Agricultural Sciences
Southern Swedish Forest Research Centre

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Abstract

Poplars hold great potential for bionenergy production. However in boreal forests where land for establishing these plantations is abundant poplars encounter challenges in establishment. One of these challenges is aluminum (Al) - one of the major factors inhibiting root growth, thus reducing water and nutrient uptake and slowing growth. Forest soils normally have low pH and thus higher levels of aluminum ions (Al3+ and hydroxides). However there is evidence of Al tolerance among *Populus* and if succsessfully identified, could be able to grow on forest sites. The aim of the study is to evaluate growth response of *Populus* hybrids to different of aluminum concentrations and contribute to the development of reliable tools for selection of Al-tolerant poplars. In this study, growth responses of greenhouse-grown poplar (P. trichocarpa hybrids) were monitored in relation to changes in Al concentrations in the rhizosphere. The differences in Al sensitivity were identified by measurements of relative shoot growth, root biomass and supported by staining with hematoxylin for root damages. The results identified several poplar clones with high or low tolerance to Al. The findings suggest that identification of Al tolerant *Populus* clones requires collection of more than one type of data. What is more, further experimentation is required to fully confirm that the selected clones are truly capable of establishment on forest sites with investigation of those sites soil properties.

Keywords: Populus; Aluminum; hematoxylin staining; clonal variation; soil pH; short-rotation forestry;

Summary

Dėl klimato kaitos pramonė bando atsisakyti iškastinio kuro ir pereiti prie bioenergijos, pagamintos iš medžio biomasės. Vienas iš sprendimų šiai paklausai patenkinti yra greitai augančių tuopų (*Populus* spp.) plantacijų naudojimas. Šiandiena bioenergijos plantacijos dažniausiai būna apleistose žemės ūkio paskirties žemėse, tačiau yra galimybė išplėsti šias plantacijas į miško žemę, kurios yra gausu borealinėse ir hemiborealinėse zonose visoje Europoje ir Skandinavijoje. Ši idėja gali atrodyti patraukli miško savininkams, nes vieni tuopų hibridai elniams žvėrims yra mažiau palankūs maisto šaltinis negu kiti. Nors, vis dar yra kitų kliūčių, trukdančių sėkmingai įsitvirtinti tupoms miško žemėse. Viena didžiausių problemų kyla dėl toksinio ir augimą slopinančio aliuminio (Al) lygio, kurio gali būti miško dirvožemyje. Esant rūgštinėms dirvožemio sąlygoms, Al pažeidžia tuopų šaknis, mažina augalų augimą ir neleidžia tuopų daigams įsitvirtinti. Tačiau Al tolerancija galima rasti ir kai kuriuose tuopų hibriduose. Šiuo tyrimu bandoma nustatyti šiuos genotipus, veikiant tuopų rūšis įvairiose Al koncentracijose, siekiant atrasti tolerantiškus genotipus, turinčius didelį potencialą augti miško žemėse. Be to, aptariu tinkamus metodus Al tolerantiškų tuopų augalų paieškose ir tolimesnes įžvalgas greitai augančių tuopų plantacijų sodinimui miško dirvožemyje.

Contents

Tables and Figures

1. Introduction

Poplars, their potential in biomass production

Due to the world‘s increasing demand of forest products, fast-growing tree species are becoming more favoured as alternatives for short rotation forestry (Bona et al., 2008). Hybrid aspen (*P. tremula* × *tremuloides*) and poplar (*P. trichocarpa* hybrids) are often used in bioenergy plantations. *Populus* L. is a genus covering about 30 species of poplars, aspens and cottonwoods, which are widely distributed over the northern hemisphere and planted in many parts of the world (Stettler et al., 1996). *Populus* is most commonly planted on abandoned agricultural land (Christersson, 2008), forest land (Bona et al., 2008) as well as floodplains (Pallardy et al., 2003). Usually these type of sites consist of plantations of *P.* × *wettsteinii* and *P. trichocarpa* and their various hybrids (Tullus, 2005; Christersson, 2008; Tullus et. al 2011).

Poplars, and especially their hybrids, are known for their rapid growth rate (Anderson et. al 1983; Ranney et. al 1987). Moreover there is an increasing interest of forest owners in poplar wood to be used not only energy wood, but also a wide range of wood products like industrial roundwood, poles, pulp and paper, plywood, veneer, sawn timber, packing crates, pallets and other services (Ball et al., 2005). Furthermore with use in short-rotation poplar as a more effective step-in for fossils fuels in energy production (Vitousek, 1991). Moreover a role for poplar plantations in carbon sequestration schemes is likely (Rytter, 2012). A well-developed root system in poplar plantations show promise to act as a filter, purifying polluted water, as the roots can pick up the phosphorous and nitrate ions from the water (Christennson, 2010).

Populus trichocarpa (and it’s hybrids) are nutrient demanding plants and suffer growth reductions in more acidic soils, when the pH in the soil is lower than 5 (Bergstedt, 1981). Low soil pH can negatively influence plant growth in several ways, for example, increased plant mortality caused by the excess of protons (H+) (Driscoll et. al 2001; Bolan et. al 2003). Manganese and especially high levels of aluminum can inhibit water uptake, and nutrient deficiencies of essential nutrients

such as phosphorus, calcium, and magnesium, which are essential for plants and especially *Populus* (Marschner 1991; Ashman and Puri 2008; Fageria and Baligar, 2008).

Aluminum as a limiting factor for poplar cultivation in forest soil

Aluminum (Al) is the most abundant metal on earth, commonly found in bauxite, it is the third most abundant element in the earth's crust (Bojórquez-Quintal et al., 2017). Despite being abundant in the soil and always available to plants, Al serves no exact biological purpose (Poschenrieder et al., 2008). Al in the soil is mainly found in the form of a minerals and oxides however, in liquid solutions and at different pH levels, Al hydrolyses water molecules to form aluminum hydroxide. Furthermore total Al concentration in the soil and the form of Al mostly depends on the pH and the chemical environment of the solution (Kisnierienė and Lapeikaitė, 2015). At a low pH (about 4.3) trivalent aluminum (Al^{3+}) is the most abundant form and has the greatest impact on plant growth.

At high concentrations, Al ions reduce nutrient availability in soils, cause damage to plant cells and thus inhibit plant growth. The mechanisms of Al-toxicity involve the cell wall and plasma membrane in the roots (Bojórquez-Quintal et al., 2017). Al modifies the cell's structure, as well as the nearby ionic medium to wall, both disturbing the transport of ions and cause an improper balance of nutrients (Bojórquez-Quintal et al., 2017). Furthermore Al can affect the root's symplast (Tokizawa et. al, 2015), apoplast (Delhaize et. al, 2007) and DNA in cells of plant roots (Sade et. al, 2016). The first symptom of Al toxicity in plants is the sudden inhibition of root elongation (Sivaguru and Horst, 1998; Bojórquez-Quintal et al., 2017). By limiting root development in crop plants increases the risk of drought (Yang *et. al*, 2013).

However, when administered at low concentrations, growth stimulations induced by Al are observed frequently in plants, which have adapted to acidic soils conditions (Pilon-Smiths et. al, 2009). In hyperraccumulator plants, that can accumulate extraordinarily high amounts of heavy metals, Al can stimulate or have no effect on nutrient uptake (Bojórquez-Quintal et al., 2017). Some plants have rely on other nutrient uptake like phosphorous, nitrogen and potassium to be stimulated by Al induced root growth (Osaki et. al, 1997). The concentration of Al may be a major factor filtering species composition on acid soils in favouring establishment of Al-resistant plants (J. Balkovič et al., 2014). This poses a challenge in establishing fast-growing poplar plantations, especially P. trichocarpa

(and their hybrids). If Al tolerance is the cause of P. trichocarpa hybrids sensitivity to low pH, genotypes with high tolerance to Al could be used at forest sites.

Al tolerance in *Populus*

Quaking aspen (*Populus* tremuloides L.), which has been suggested to have acidic soil resistance (Naik et. al 2009), and aspen (*Populus tremula* L.) are repeatedly found in boreal forests where the soil pH is low (Böhlenius et al., 2018). There is substantially more forest land available compared than agricultural land. In these lands, plantations of poplar hybrids species offer an alternative for biomass production (Böhlenius et al., 2018). However, the boreal and nemo-boreal forests, that cover large parts of Russia, China, Scandinavia and the North American continent is naturally acidic with a soil pH range of 3.7 and 6.4 (Bona et al., 2008; Böhlenius et al., 2018).

Other reports suggest that *P. trichocarpa* and their hybrids are sensitive to low pH and display an optimum growth when the soil pH levels are between 5.5 and 6.5 (Bergstedt 1981; Jobling 1990). With decreasing soil pH the bioavailability of aluminum and its solubilization rises, which leads to inhibition of plant growth by damaging roots and halting nutrient uptake, which disturbs the establishment (Christersson, 2008; Böhlenius et al., 2018).

Aluminum minerals are common in Podzolic soils which are generally infertile and are physically limiting soils for productive use. Furthermore, Al^{3+} is, in general, toxic to poplars and its solubility dramatically increases at pH values < 4.5 (Ashman and Puri, 2008).

Challenges and opportunities of establishing *Populus* on forest sites

Hybrid aspen (*Populus tremula x tremuloides*) has been identified to be one of the fastest growing tree species in Europe. The hybrid has been established in the early 20th century and in several researches has proven to produce a higher capacity of biomass than either of its parent species in the first 20-30 years (Rytter, 2006). Their Fast root growth allows them to reach contact to soil water and nutrients much faster. This suggests that with appropriate soil conditions fast growth will also have a positive impact on plants reaching browsing free heights

faster than other broadleaf species, potentially making the use of fencing against ungulates an avoidable expense if necessary.

Furthermore considerably more forest land is available than agricultural land. This becomes especially appealing when for example considering countries like Sweden where forests cover is about 69% and agricultural land being about 7,5% or Russia - almost 50% forest land compared to about 13,2% agricultural (The world bank, 2016). Establishing poplar plantations on forest land has the advantage that biomass production on this land would not interfere with food production (Böhlenius et al. 2018). What is more prior research indicates that young hybrid aspen stands can support relatively diverse and distinctive bird communities (Lindbladh et al., 2014) granting more ecosystem services besides carbon sequestration.

However hybrid poplar plantations established at forest sites, as opposed to agricultural lands, pose challenges in terms of soil fertility and tree nutrition. Forest soils do not have long history of fertilizer amendments the way agricultural soils do (Vande Walle et. al, 2007) and are often less fertile, at least in the boreal zone (Bilodeau-Gauthier et al., 2010). In addition, forest sites present challenges with control of competitive vegetation. Plantations of hybrid aspen are susceptible to extensive grazing from moose and deer, and thus require expensive fencing (Böhlenius et al., 2018) and further maintenance which increases costs.

Despite *P. trichocarpa* (and its hybrids) suffering from declines in development when the soil pH is <5, poplar hybrids are less favoured by moose, deer and elk (Lof et al., 2010). Nonetheless, extensive liming in order to raise the soil pH would be required to ensure successful establishment, which on larger scales may result in high costs and thus prove less profitable. Hence both species face challenges when it comes to fast-growing tree plantations either from ungulate grazing or unsuitable soil conditions. Nonetheless with extensive knowledge of various poplar hybrids being more or less tolerant to Al, the option of identifying them could make them more suitable for forest land. This might outweigh all the challenges and make hybrid poplar plantations on forest land perhaps attractive to forest owners.

The establishment of hybrid poplars on forest sites can be further strengthened if the mechanism that induce Al-tolerance could be screened and identified. The mechanism of Al sensitivity and resistance has been well documented for agricultural crops and tree species (McCormick et al., 1978) and for genotypes of poplar species (Steiner et al., 1984). These plants show great diversity in their response to Al.

Populus exudes organic acids that inhibit Al uptake by roots. Root growth measures and staining with hematoxylin have been the most used techniques because they have produced consistent results (Lima Echart et al., 2002). The indicator dye hematoxylin has been proven useful in identifying Al-tolerant genotypes in a many species like barley, wheat, tomato, teak and poplar (Bona et al., 1998; Lima Echart et al., 2002; Polle et al., 1978) Prior research proposes Al screening with hematoxylin to be a proficient and non destructive method of identifying root damage by Al as similar results have also been found in barley (Smith et al., 2011), wheat (Polle et al., 1978) and other plant species. The hematoxylin detect both accumulation and exclusion of A1 in root tips (Polle et al. 1978).

Study aims

The aim of the study is to evaluate growth responses of *Populus* hybrids to different aluminum concentrations. The goal is to increase the knowledge about the factors and processes affecting the success or failure of poplar cultivation in forest land. This study aims to confirm the earlier results by Böhlenius et al., (2018) that there is a variation in Al resistance both among and within the *Populus* species. Furthermore to contribute to the development of reliable tools for selection of Al-tolerant poplar genotypes.

The research objectives are:

- In a controlled environment, identify the most Al tolerant and susceptible clones based on the growth responses to different Al concentrations.

- Compare the results obtained through the different methods of growth measures and staining.

2. Materials and Methods

Plant material

For the experiment, 21 poplar clones and 2 hybrid aspen clones were selected **(Table 1)**. These clones were chosen as they are commercially available and with variable genetic backgrounds.

Table 1. *Overview of the Populus and Hybrid aspen clones used in the Al tolerance experiment, showing clone number, genotype, commercial name and country of supply.*

Clone nr.	Genotype	Commercial clone name	Supply country
1	P. trichocarpa	*No name*	Ekebo, Sweden
6	P. maximowiczii x P. trichocarpa	*No name*	Ekebo, Sweden
99	P. maximowiczii x P. trichocarpa	OP42	Ekebo, Sweden
266	P. deltoides x P. nigra	*No name*	Italy
306	P.trichocarpa	*No name*	Ekebo, Sweden
311	P.trichocarpa	*No name*	Ekebo, Sweden
312	P.trichocarpa	*No name*	Ekebo, Sweden
350	P.trichocarpa	*No name*	Ekebo, Sweden
405	P.trichocarpa	*No name*	Latvia
406	P.trichocarpa	*No name*	Latvia
407	P.trichocarpa	*No name*	Latvia
527	P. deltoides x P. nigra	*No name*	Minnesota
534	P. deltoides x P. nigra	*No name*	Minnesota
536	P. trichocarpa x P. deltoides	Dx	Minnesota
551	P. nigra x P. maximowiczii	Max2	Germany
557		Max5	Germany
L93		SweeTree Technology	British Columbia
L130		SweeTree Technology	British Columbia
L192		SweeTree Technology	British Columbia
L200		SweeTree Technology	British Columbia
L214		SweeTree Technology	British Columbia
KI0001	Populus tremula L. x tremuloides	*No name*	Ekebo, Sweden
KI0002	Populus tremula L. x tremuloides	*No name*	Ekebo, Sweden

Experimental design, growth conditions and data collection for analysis of Al-sensitivity

The prepared poplar cuttings were approximately 10 cm long and 5-10mm in diameter, stored in cool temperatures to maintain dormancy until the beginning of the experiment. The poplar cuttings had two buds (at the top and bottom of the cuttings). The container-grown hybrid aspen seedlings (30±40 cm tall, 3.5±4.0 mm root collar diameter) were root washed before planting. Plastic trays of 0,5 -litre pots (15 pots per tray) containing siliceous quartz sand (0.45 mm grain size) and water permeable agro-cloth at the bottom to prevent the sand from escaping were prepared for the cuttings.

The experiment consisted of eight blocks, each containing five Al-treatments: a Al-0, control; Al-100, 100mg/l $AlCl_3$; Al-130, 130mg/l $AlCl_3$; Al-160, 160mg/l $AlCl_3$ and Al-200, 200mg/l $AlCl_3$. Each treatment had one plant per clone (21 poplar cuttings and two of the hybrid aspen seedlings) randomly planted in each treatment (Figures 1 and 2). In total 840 poplar cuttings and 80 hybrid aspen seedlings were used for all eight blocks

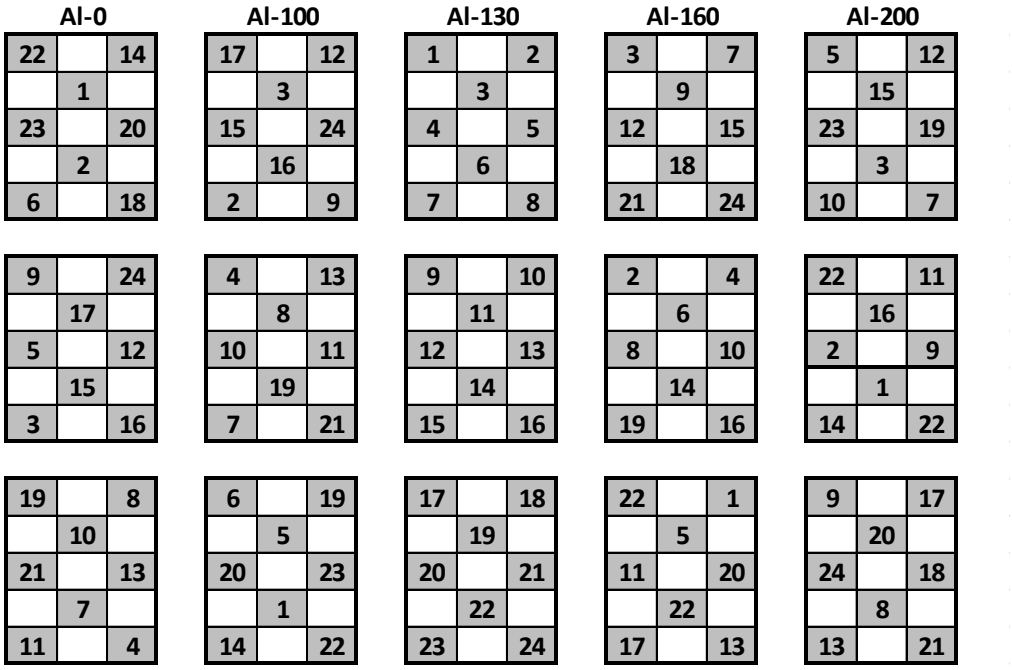

Figure 1. *A schematic displaying the layout of the clones in one block. The numbers indicate a position where a clone could be planted.*

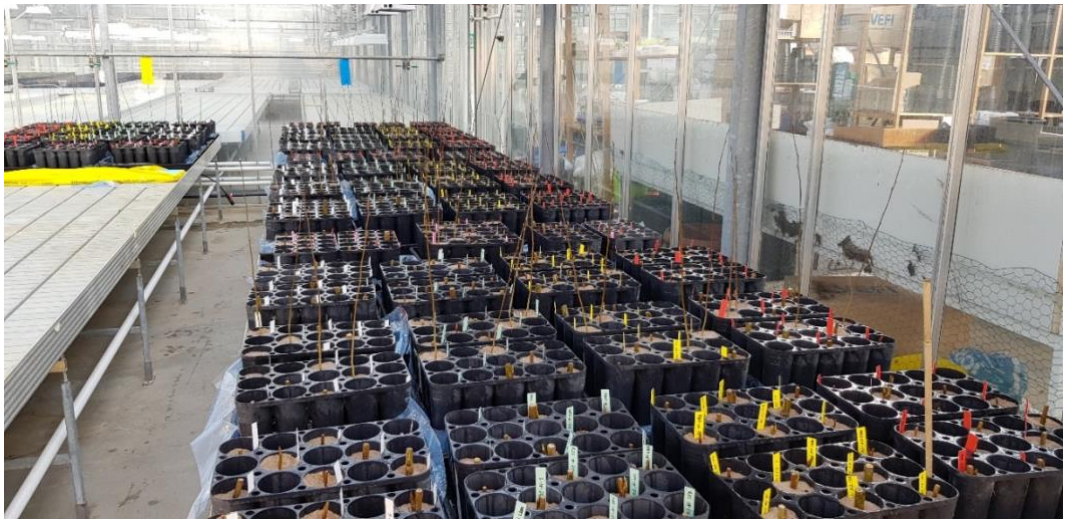

Figure 2. *Photot of the Populus and Hybrid aspen cuttings in a greenhouse environment. The tags indicate block and clone number. Different colours specify the Al treatment applied: White: Control; Green: Al-100; Yellow: Al-130; Red: Al-160 and Pink: Al-200.*

Before applying the Al-treatments, the plants were kept in the greenhouse with regular, daytime light. During this period the plants were regularly irrigated with nutrient solution which was prepared by dissolving fertilizer (0.37 g Superba rod and 0.37 g calcinit YARA Liva per litre (L) deionized water), adjusting the pH to 4.2 with hydrochloric acid (HCl). The irrigation process was conducted daily for about month in order to allow the plants to produce roots and grow measurable shots.

Before the Al treatments were started, the height of each shoot was recorded. The Al treatment was performed by irrigating the plants with nutrient solution supplemented with $AlCl_3$ to achieve according concentrations of Al: Control – 0mg/L; Al-100 – 100mg/L; Al-130 – 130mg/L; Al-160 – 160mg/L; Al-200 - 200mg/L. The pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl) to 4.2. After eight weeks of Al treatment secondary shoot height measurements were taken to calculate shoot height growth under influence of Al. Furthermore, the roots were harvested and dried for 48 hours to weigh dry root biomass.

Calculations and statistical analysis

Shoot growth was calculated by deducting the first recorded height measurement from the second. Relative shoot height increment for each clone was calculated by dividing each growth value by the mean value of that clones control shoot height values. This value for the mean control of a clone gave a value of 1, while Al treatment shoot height growth value were either above or below 1. Thus, a mean value >1 indicated that the treatment stimulated the growth, and a mean value <1 reduced the shoot height growth. In order to compare the inhibition of root growth by Al, relative root biomass of the plants treated with Al was calculated by dividing the root biomass of individual plants by the mean of the corresponding untreated plants of the same genotype. To simplify the results mean values of all 8 blocks per clone were calculated.

Statistical analysis was done using R version 4.0.2 (R CoreTeam) with mixed models following the 'lme4' package. To evaluate differences among treatments, I used Tukey´s HSD as a post-hoc test, implemented in the "emmeans" R package. A p-value of ≤ 0.05 was used as the cut off for statistical significance. Residuals were inspected and showed normal distributions with no high-leverage outliers using the mixed model.

Hematoxylin staining

On the basis of shoot height growth calculations (Figure 4): three tolerant and two susceptible poplar clones were chosen to be stained with hemaxotylin after the treatment to identify potential root damage done by Al treatments. The extracted poplar cutting roots were washed with deionized water for 10 minutes and stained with 1g/L hematoxylin (Sigma±Aldrich, Seelze, Germany) and 0.1g/l of KIO3 (Riedel-de HaeÈn, St: Louis USA) for 10 minutes. After staining, roots were washed again in deionized water. The degree of staining was evaluated by visual inspection and photos were taken.

3. Results

Relative shoot height increment.

There was a variation in relative height growth response between the clones (Figure 3). For the different Al concentrations, a different growth reduction was found with Al-100 (Figure 3-A) showing a low growth reduction (40 to 100%) and Al-200 (Figure 3-D) having a large growth reduction (10 to 60%). For the other Al treatments e.i. Al-130 and Al-160, the variations were larger with Al-130 (Figure 3-B) and Al-160 (Figure 3-C) displaying a growth reduction of 20 to 100% and 18 to 100%, respectively and suitable concentrations for selecting Al sensitive and tolerant poplar clones. When sorting from sensitive to tolerant within Al-160mg/l, three tolerant and two susceptible clone candidates could be identified (Figure 4).

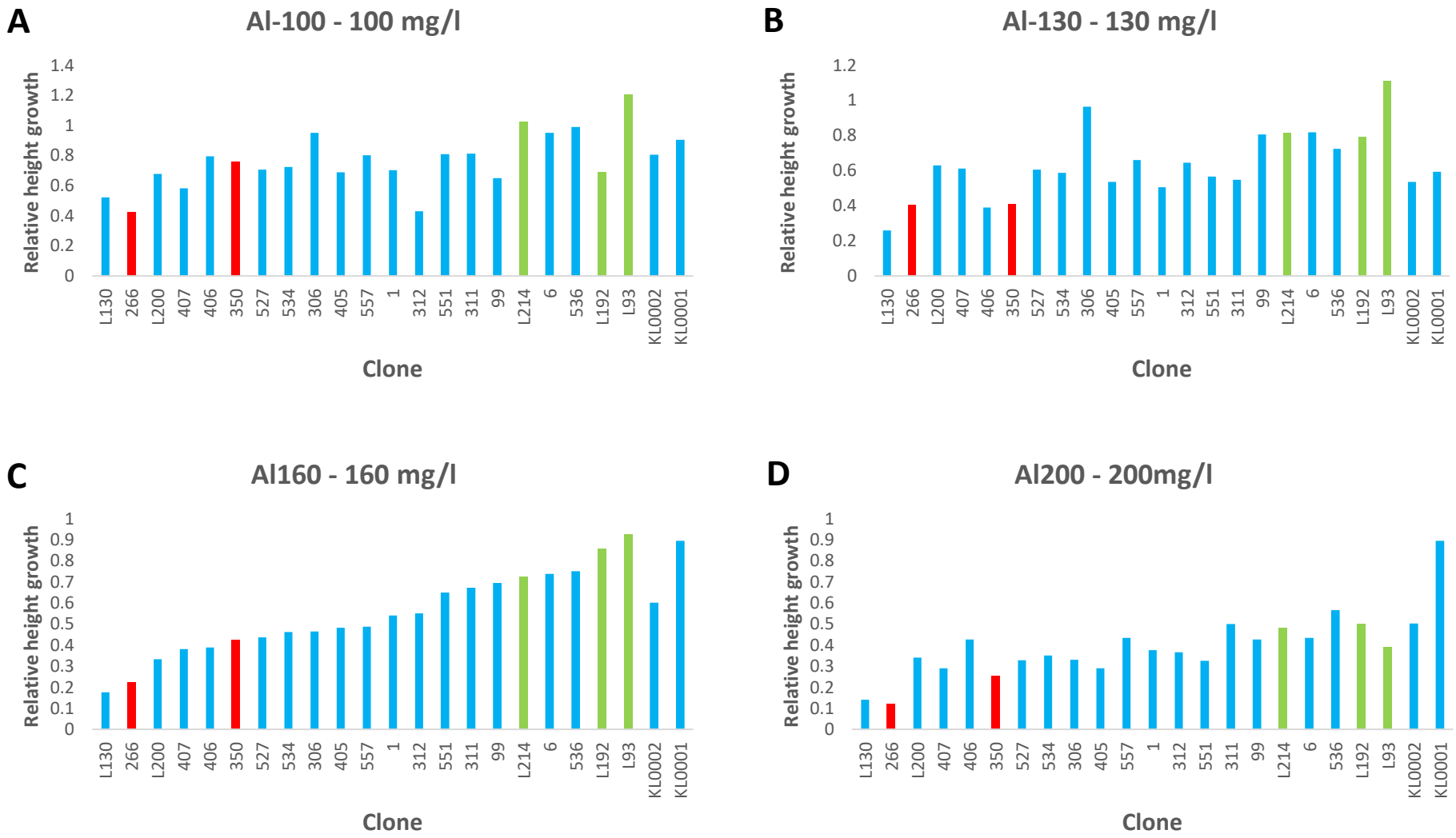

Figure 3. *Histograms displaying the relative shoot height growth distribution for Populus clones, growing in four Al concentrations 100; 130; 160; 200 mg/L. The selected tolerant and susceptible clones are highlighted in green and red, respectively.*

One of the three tolerant clones – L93 displayed a 10% better relative shoot growth in Al-130 and a 7% decrease at Al-160 Al treatments. Clone L214 showed a 19% growth decrease at Al-130 and a 28% growth decrease in treatment Al-160. Clone L192 had a 21% shoot growth decrease in treatment Al-130 and 14% decrease in Al-160. The susceptible clones 350 and 266 displayed a growth reduction to 88% at Al concentration 130, 160 and 200 mg/l (Figure 4). The growth decrease for these five clones in Al-200 ranged from to 88%. Thus, these clones were selected for staining with hematoxylin (Figure 7).

Statistical analyses showed tolerant clones (L93; L192: L214) having significantly different results at Al-130 and Al-160 from the susceptible clones (266 and 350). Tolerant clone L93 and susceptible clone 266 displayed complete significant difference between them at Al-130 and Al-160.

Statistical analyses for Al concentrations 100 and 200 mg/l showed no significant differences between the clones. To obtain further evidence for the observed Al tolerance or sensitivity among the tested *Populus* genotypes, root staining with hematoxylin was performed. Root samples from the five selected clones that showed either Al tolerance or susceptibility by relative shoot growth were chosen (Figure 7).

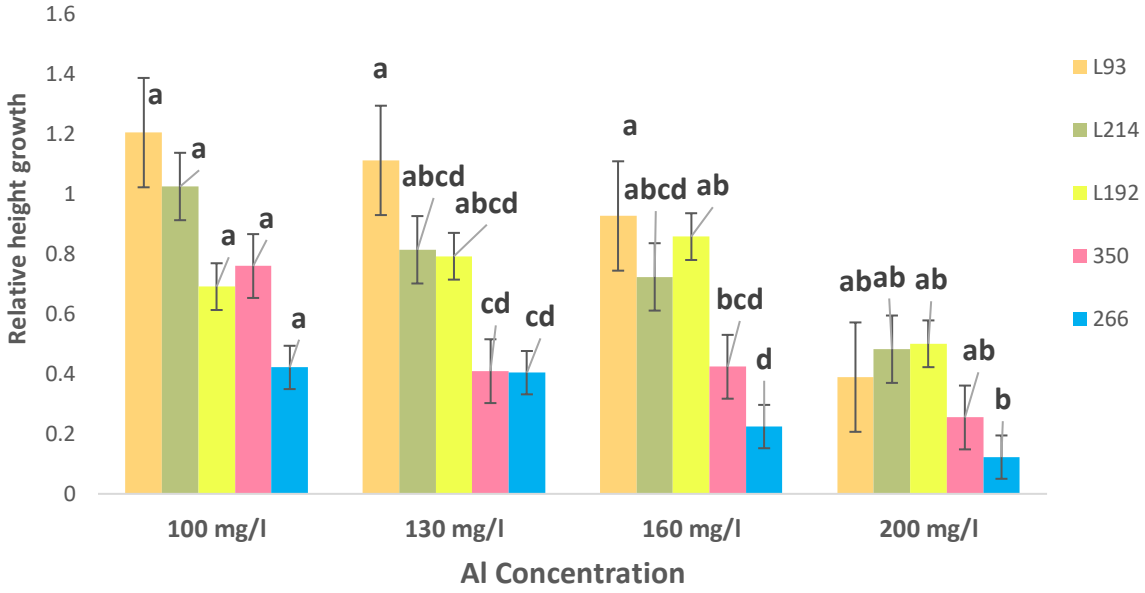

Figure 4. *Histogram showing selected potentially Al tolerant and susceptible Populus clones, based on their relative shoot height growth at four Al Concentrations 100; 130; 160; 200 mg/L. Error bars show standard errors. Bars labeled with different letters are significantly different at the p = 0.05 level within each Al concentration treatment (Tukey's HSD test α.=0,05)*

Relative root biomass and data analysis

Root biomass increment (Figure 5) varied between the clones. For the different Al concentrations, a different biomass reduction was found with Al-100 (Figure 5-A) showing a wide growth response ranging from -71% to 183 % biomass. For treatment Al-200 (Figure 5-D) having a wide relative biomass range from -79% to 163%. For the other Al treatments e.i. Al-130 and Al-160, the variations were also large with Al-130 from -76% to 191% (Figure 5-B). Treatment Al-160 (Figure 5-C) displayed the largest root biomass range from a -92% to a 213%. Based on these values, three tolerant and two susceptible clone candidates could be identified (Figure 6).

Clones 406 and L200 were considered the most affected by all Al treatments, displaying reduced root biomass from 80 to 47 % throughout the treatments. Clones 306, L192 and especially 311 displayed increased relative root growth under Al treatments. Clones – L192 displayed 134% relative root biomass in Al-160. Clone 306 had 151% root biomass at Al-130 and a 136% in treatment Al-160. Clone 311 had the highest relative root biomass with a 91% root biomass in treatment Al-130 and a 213% increase in Al-160 compared to its untreated clones. In summary these three clones e.i. 306, 311 and L192 increased their relative root growth more than their untreated clones.

Statistical analysis of the clones identified as sensitive or tolerant by relative root biomass showed that there is significant differences between the selected tolerant and susceptible clones except at Al-100 treatment (Figure 6). By applying statistical analysis for treatments Al-130; Al-160 and Al-200, clone 311 statistically significant differences from clones 406 and L200, meaning Al treatments influence root growth at all used Al concentrations.

Relative root biomass of clones selected in reference to relative shoot growth.

For the tolerant clones selected based on relative shoot growth (L93, L192 and L214) showed a reduction in relative root biomass. Clone L93 displayed a

reduction of 6% for Al-160 (Figure 5 C) to 46 % in Al-200 (Figure 5 D) treatment. Furthermore, Clone L214 displayed lower root biomass range from 35% in Al-200 (Figure 5 D) to 55% reduction in Al-160 (Figure 5 C). Susceptible clones, selected by relative height growth also displayed lower root biomass. Clone 266 had 68% lower root biomass in Al-130 (Figure 5 B), while clones 350 displayed a 73 % lower relative root growth in Al-130 (Figure 5 B).

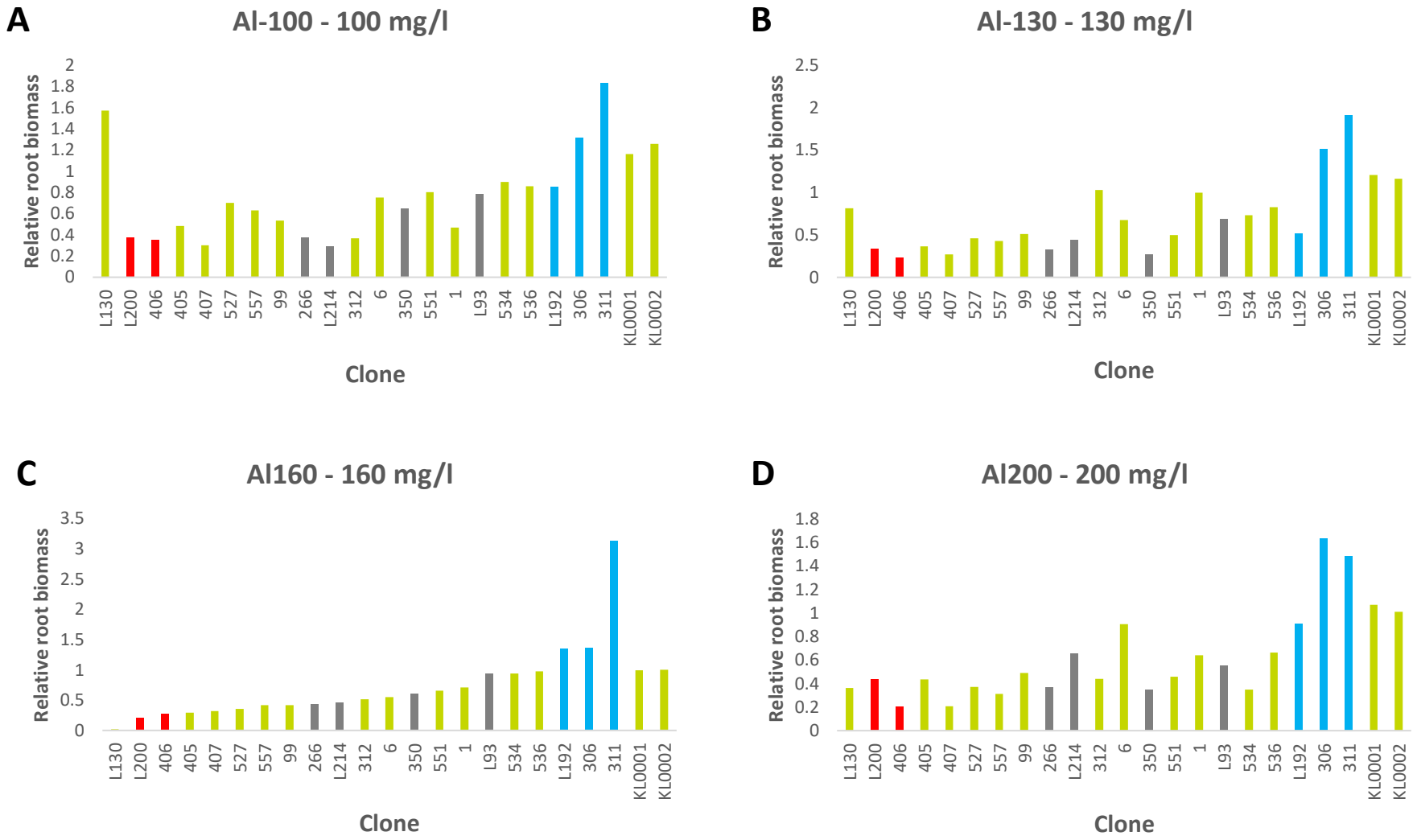

Figure 5. *Histograms displaying the relative root biomass distribution for Populus clones, growing in four Al concentrations 100; 130; 160; 200 mg/L. The selected tolerant and susceptible clones are highlighted in blue and red, respectively and clones selected in reference to relative shoot growth results highlighted in grey..*

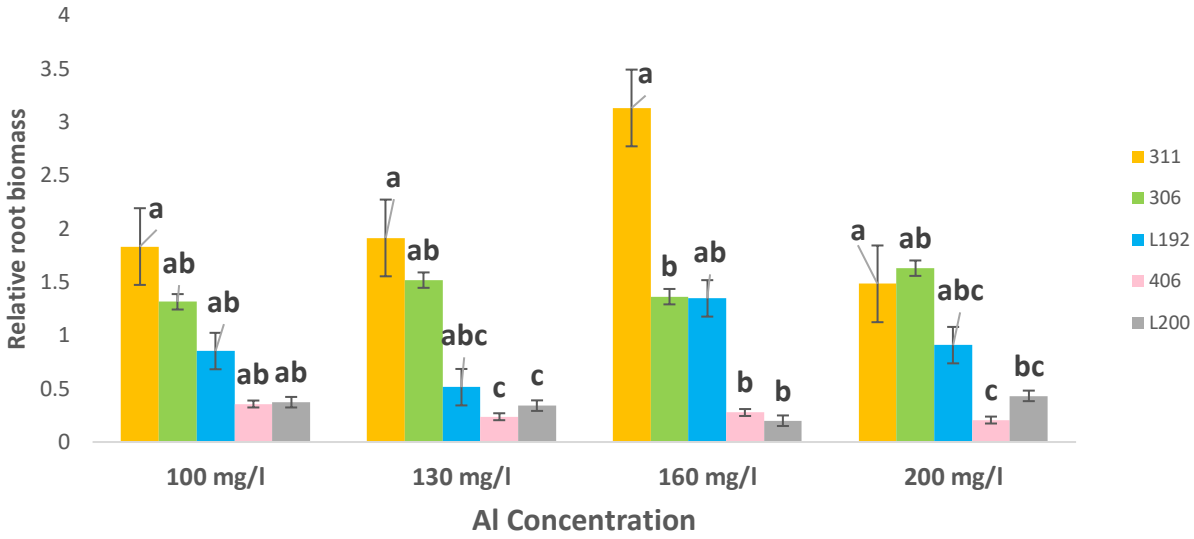

Figure 6. *Histogram showing selected potentially Al tolerant and susceptible Populus clones, based on their relative root biomass at four Al Concentrations 100; 130; 160; 200 mg/L. Error bars show standard errors. Bars labeled with different letters are significantly different at the p = 0.05 level within each Al concentration treatment (Tukey's HSD test α.=0,05)*

Root staining with hematoxylin

To complement the observed Al tolerance and sensitivity using shoot growth, a selection of tolerant and succeptible poplar clones (266; 350; L93; L192; L214) roots were selected and stained with hematoxylin to detect root damage (Figure 7). Roots that display less staining of hematoxylin can be related to higher Al tolerance. Al sensitive poplar genotypes exhibit inhibition of root growth with the root apex being the most sensitive region to Al induced stress (Figure 7 B, C). Hematoxylin staining increased with higher Al concentrations (Figure 7 C, L, O). Staining showed that clone L93 and L192 produced less hematoxylin staining throughout the Al concentrations (Figure 7 I, L) suggesting Al tolerance.

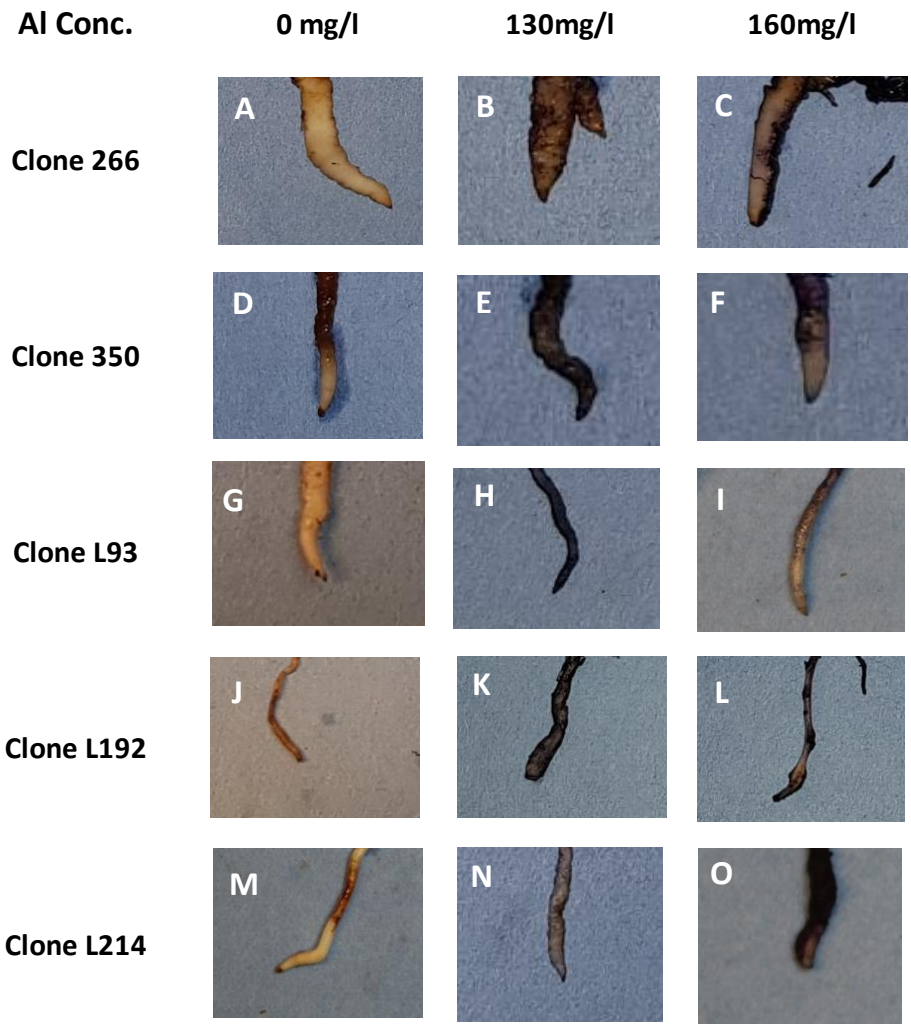

Figure 7*. Hematoxylin staining of poplar roots of control; Al-130 and Al-160 treatments. Dark staining indicates that the roots are damaged and low staining indicates that there is no root damage. Selected by relative shoot growth: Clones 266 and 350 are sensitive and clones L93, L192 and L214 are tolerant to aluminum*

4. Discussion

Growth responses to Al concentrations

Growth reductions are often detected when Al sensitive plant species are exposed to Al (Böhlenius et. al, 2018). In controlled experiments, it is important to consider which range of the Al concentration allow separation of tolerant and sensitive plants. The findings of my study indicate that Al-100 treatment was too low and Al-200 treatment was too high to allow separation (Figure 4 and 6). However Al-130 and Al-160 were suitable concentrations to use. These findings are in accordance with the findings by Böhlenius et al. (2018) who showed that lower concentrations (10; 30; 50; 100 mg/L) did not induce significant response differences among poplar clones (Böhlenius et al., 2018). However, in some studies with poplars concentrations as low as 50 mg/l have been used with success (Naik et al., 2009). This could be due to the fact that plants in various experiments were of different origins and having different tolerances to Al, or that the type of nutrient supplements used before the application of Al could also influence different growth responses in the poplar clones.

The reasoning on this is that the poplar growth responses in Al-100 and Al-200 are similar to the responses in the study by Böhlenius et al. (2018) where nutrient supplements and plant material used were the same. Furthermore the methods used in earlier experiments use similar growing conditions conducted under greenhouse conditions. Data collection in earlier research varies with analysis of dry plant, leaf, shoot or root biomass measurements and shoot growth or root damage screening with hematoxylin. However research suggests data collection of root biomass and shoot growth being the most promising, as it was used for this experiment (Böhlenius et al., 2018).

Al tolerance by root growth versus shoot growth

The results of this research can be viewed from two parts: tolerant and susceptible clone selection by shoot growth or by root biomass. Clones that responded with higher shoot growth had relatively less root biomass than their untreated clones. This shows a stressful reaction to Al and induce less root growth and more shoot growth. On the other hand clones that reacted with higher root biomass may show a delayed shoot growth response and more root establishment. Tolerant poplar clones with more root mass may be more suitable for forest soils as they would first establish a succesful root system for nutrient uptake and begin shoot growth in later vegetative periods. Additionally, root colonization by symbiotic arbuscular mycorrhizal fungi increases plant resistance to acidity and phytotoxic levels of Al in the soil environment (Seguel et al., 2013). Nonetheless its worth mentioning that untreated poplar clones had less root biomass with a lower standart deviations than the clones in the Al treatments (See appendix 1). This is consistent with other findings that have showed that stress can increase variation of measured variables for instance chemical response (Böhlenius et al., 2018). Moreover, these results indicate that there is a plasticity in the resonance to Al within a specific genotype. This means that clones with a large variation in root biomass could be more adapted to grow at variable site conditions as they are capable to respond to different environmental and soil conditions. On the other hand it might be due to the clones just having poor root growth and not as a stress responce to Al (Böhlenius et al., 2018). Clones that have poor rooting capacity might be unfit not because of their Al tolerance or sensitivity but because of the lack of root growth, making establishment on forest sites more difficult.

Nonetheless the results of my experiments indicate that it is very important to consider what kind of data is collected, because the method affects the results. Screening of Al tolerance by both and root growth measurements results in complementary data that supports each other.

Mechanisms behind Al tolerance

This study revealed *Populus* clones that were highly tolerant to Al, and others that were very sensitive. However some tree species have mechanisms that prevent

these damages by Al, for example quaking aspen (Naik et al., 2009; Böhlenius et al., 2018). Other studies reveal that poplar genotypes like *P. tremuloides and P. trichocarpa* Al stimulates perculation of organic acids like oxalate, malate and citrate (Naik et al., 2009) and stimulates release of oxalate and citrate in *P. tremula* (Qin et al., 2007). These organic acids effectively chelates Al, thereby detoxify it in the rhizosphere and supporting Al tolerance (Böhlenius et al., 2018). Furthermore arbuscular mycorrhizal fungi in host plants contribute to detoxifying Al in the rhizosphere and consider to alter Al bioavailability (Seguel et al., 2013) The two hybrid aspen clones in this study (Figure 3; 5) displayed prominent Al tolerance, similarily as in other Al sensitivity studies (Böhlenius et al., 2018). Moreover, genes that are involved in cell wall modification, oxidative stress and ion transport have been shown to be up-regulated during Al treatments of aspen (Grisel et. al, 2010). What is interesting that recorded genes ALS3 and MATE which encode oxalate and citrate, responsible for Al detoxification and can be found in hybrid aspen and also *P. trichocarpa* (Böhlenius et al., 2018). These genes and their activity in poplars might support tolerance of Al in poplars by stimulating exudation of organic acids, possibly citrate and prevent Al uptake by the roots and avoiding root damages.

The tolerance to Al can also be explained by forest trees adapting to naturally acidic soil conditions and developing defence mechanisms that enables them to tolerate Al (Böhlenius et al., 2018). Therefore, the provenance of the tree species, may play a role in Al tolerance. If so my suggestion would be to attempt to further study clone cultivars of poplar species found on forest sites in order to identify natural Al tolerance.

Challenges of poplar plantations on forest sites

If poplars are to be grown on forest land, there are more aspects to adress than just selecting Al tolerant clones. Prior experiments by Böhlenius et al. (2018) revealed that *Populus* plantations on forest sites face also other challenges that Al toxicity increases. Poplars are a nutrient demanding are trees and suffer growth reductions in more acidic soils. New root growth following establishment is key for sucssessful seedling growth and Al toxicity inhibits root growth. Furthermore Al toxicity constricts rooting depth and root branching, preventing access to subsoil nutrients (Böhlenius et al., 2018). This would make soil preparation neccessary to provide seedlings access to nutrients in lower layers and to initiate growth on forest land. Moreover earlier field studies recorded that for the living poplar plants, the leading shoot of poplars was often severely damaged or dead, probably

due to drought (Böhlenius et al., 2018). Nonetheless, conducting forest soil sampling to identify soil pH and Al concentration properties for more attractive forest sites for poplar plantations could be helpful.

Conclusions and insights on future Al tolerance studies

While this study has successfully identified poplar clones that are potentially Al tolerant, these results do not confirm that the selected clones will successfully establish in forest sites. As there are many factors that jointly influence the success of establishment of poplar plantations on forest sites, further studies are important. This study confirmed that shoot height growth and also root biomass are both important to monitor in order to identify Al tolerant or sensitive clones. Furthermore the use of hematoxylin staining to detect Al-tolerant genotypes can be an important tool to help identify Al tolerant clones and establish them on forest sites (Böhlenius et al., 2018).

Another issue to address is root growth and penetration into forest soils, which poses a challenge to seedling establishment. I believe it is necessary to conduct soil studies in order to identify forest soil pH and Al concentration before the planting of seedlings. Furthermore perhaps expanding the experiment by using different soil types, as sand which was used in this experiment is much more permeable than soils found on forest sites.

Nonetheless I do believe the results from this, prior and future studies will help to find sustainable solutions to successfully establishing fast-growing *Populus* plantations on forest sites which are not only abundant on Sweden, but all the boreal and hemiboreal zones that cover Scandinavia as well as Europe.

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Acknowledgements

I would like to thank Henrik Böhlenius for being my supervisor, for arranging the working environment and support, thus making this study possible. A huge thanks to Håkan Asp for supplying the necessary materials and equipment used in this study. Also I wish to thank Mateusz Liziniewicz from Skogforsk Anelie Adler from Sweetree Technology for supplying the plant material for the experiment. Finally, I would like to thank my partner Agnė Paulauskaitė, who assisted in the collection of the data for the analysis of this experiment.

Appendix 1

Table displaying average clone root biomass in grams for treatments: Control; Al-100; Al-130; Al-160 and Al-200 with number of root samples measured for each clone. Tolerant/susceptible clones, selected through relative biomass calculations are highlighted green.

Treatment	Control			Al-100			Al-130			Al-160			Al-200		
Clone nr.	Mass	StDev	No. samples	Mass	StDev	No. samples	Mass	StDev	No. samples	Mass	StDev	No. samples	Mass	StDev	No. samples
1	0,533	0,438	8	0,250	0,172	8	0,531	0,379	8	0,376	0,254	8	0,340	0,222	8
6	0,446	0,232	8	0,335	0,219	8	0,301	0,204	8	0,245	0,179	8	0,403	0,226	7
99	0,539	0,545	8	0,289	0,205	7	0,276	0,101	8	0,226	0,121	8	0,265	0,152	8
266	0,887	0,775	7	0,331	0,229	8	0,289	0,143	7	0,380	0,205	8	0,323	0,307	8
306	0,584	0,443	8	0,768	0,520	8	0,886	0,404	8	0,796	0,588	7	0,952	0,649	6
311	0,319	0,278	8	0,584	0,407	7	0,610	0,383	8	0,999	0,671	7	0,473	0,263	7
312	0,433	0,410	6	0,159	0,080	7	0,446	0,491	8	0,222	0,211	6	0,190	0,142	8
350	0,695	0,726	8	0,450	0,331	6	0,190	0,104	7	0,416	0,225	8	0,239	0,171	7
405	0,884	0,800	8	0,428	0,252	8	0,325	0,239	8	0,261	0,144	8	0,384	0,237	8
406	1,319	1,021	8	0,470	0,262	6	0,313	0,231	7	0,365	0,233	6	0,272	0,205	6
407	1,029	0,861	7	0,310	0,238	7	0,278	0,140	8	0,326	0,210	5	0,213	0,186	7
527	1,067	0,826	7	0,749	0,653	7	0,493	0,326	8	0,383	0,386	8	0,394	0,383	7
534	0,621	0,366	8	0,560	0,263	8	0,456	0,320	8	0,584	0,253	8	0,217	0,145	7
536	0,483	0,587	6	0,415	0,259	8	0,401	0,352	8	0,471	0,333	7	0,321	0,164	7
551	0,810	0,382	8	0,649	0,390	8	0,403	0,152	8	0,529	0,367	8	0,371	0,181	8
557	0,960	0,815	8	0,605	0,206	8	0,409	0,152	8	0,403	0,251	8	0,298	0,151	8
L93	0,769	0,583	7	0,607	0,466	6	0,531	0,246	7	0,722	0,543	6	0,423	0,244	4
L130	0,550	0,346	4	0,864	1,030	7	0,449	0,757	7	0,010	0,004	1	0,200	0,135	3
L192	0,350	0,278	3	0,299	0,260	7	0,180	0,095	2	0,471	0,351	7	0,318	0,203	5
L200	1,022	0,620	6	0,381	0,243	7	0,349	0,243	7	0,204	0,122	5	0,442	0,304	5
L214	0,730	0,530	5	0,211	0,111	7	0,320	0,265	4	0,330	0,182	5	0,475	0,320	5
KL0001	3,054	1,599	8	3,548	0,994	8	3,684	1,811	8	3,029	0,948	8	3,259	1,218	8
KL0002	2,320	0,559	8	2,921	1,023	8	2,703	0,828	8	2,333	0,883	8	2,345	1,282	8