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Microelement Localization In Leaves Of Populus spp. and Tolerance Mechanisms to Boron-Salt Toxicity

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by

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Abstract

Phytoremediation has been proposed as a low-cost, environmentally friendly alternative to clean highly contaminated sites. Phytoremediation using trees offer the advantage of metal immobilization (phytostabilization), high hydraulic pumping pressures, produce large plant biomass, absorption of total dissolved salts within their tissue, long life spans, less frequent irrigation and, a massive root system that helps soil attachment. Hybrid poplar varieties are long-lived, perennial, fast growing, and produce a large amount of foliage to harvest. Boron is an essential micronutrient for plants. Certain locations, such as California and Turkey, have suffered from high boron efflux from irrigation waters. It has been identified some hybrid poplar clones that can withstand elevated concentrations of boron and salinity. Most studies are based on root to shoot metal translocation mechanisms but there is little evidence of data showing detoxification strategies in the ability of tissues to tolerate high B concentrations. It is essential to investigate the compartmentation, microelement localization and tolerance/detoxification mechanisms of contaminants at tree crown and leaf tissues, at a sub-cellular level to identify the strategies and cellular structures in charge of controlling the stress reactions in previously selected hybrid tolerant populus trees. The aim in this study was to describe the contaminant allocation in leaf tissues of specialized tolerant and sensitive poplar clones utilizing different histochemical and micro-analytical methods. In the present study, multiple element contamination effects on poplar leaves were ascertained using histochemical observations and X-ray micro analytical method. In addition, foliar element concentration was measured and compared with biomass, and physical observations. Results showed that biomass but not the tree height is affected regarding the metal concentration. Most effects (i.e necrosis) and visible symptoms were observed more apparent in leaf apexes and in younger leaves than in other leaf parts. Also, leaf branch position closer to the main stem and leaf tip and margins presented more contamination symptoms. Intercellular abiotic deposits are located on a water/mineral element route and are associated to local cell wall thickenings and wart-like protrusions - both are typical stress reactions. Condensed tannins were involved in metal chelating. Antioxidant functions from phenolics were involved in metal detoxification. Pectin was a major sink for boron. At the principal

accumulation site of contaminants, coincidence of a naturally resistant tissue on the pathway of an overloaded nutrient flux, in the lower leaf blade tissues, there is a 2-3 cell layer-thick hypodermis. Hypodermis is a specialized tissue, with low cell physiological activity, and, being not involved in vital processes such as photosynthesis and assimilates transport, is an ideal site for B & salt detoxification. Stress and tolerance reactions indicate that cells in the hypodermis structure are well suited for accumulation. Cell wall thickening and various symplastic defense mechanisms were observed on both locations were the study was implemented and thus appear to be constitutive in foliage of these clones. Foliage concentrations detected several metal contaminations (noteworthy Na, B, Cl, Se). Micro analytical analysis confirmed the metal allocation as described in histochemical observations. It is distinguished a tendency from most elements to accumulate in the lower mesophyll. For several reasons, micro analytical analysis should be considered as tendencies. Better techniques should be implemented to characterize the allocation of metals in the leaf tissues, especially for Boron. Implementation of molecular methods for a better understanding of the efflux transporters should complement the results obtained in this study.

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Chapter 1: Introduction

PHYTORMEDIATION

Phytoremediation uses plants to mitigate contaminated soils and groundwater. Chaney (1983) was the first to introduce the idea of using plants that accumulate metals to selectively take out excessive waste constituents. Moreover, Brooks et al. (1977) started studies to find plants or "hyperaccumulators" that can accumulate excessive amount of metals in the plant tissues. Plants extract necessary organic and inorganic compounds found in the soil for its natural functions. Plants referred as hyperaccumulators can store large amounts of these compounds or metals for their natural metabolic processes. A complete review on hyperaccumulating plants and how they work is given by Raskin and Ensley (2000) and Rascio and Navari-Izzo (2011).

When water is extracted from the ground, plants help to lessen erosion, leaching, runoff, and restraining the movement of pollutant. Soil contaminants are absorbed by plants that can metabolize, volatilize, or store them as by-products. Additionally, the plant root may form an aerobic environment where biological activity is enhanced and metal movement is reduced (Robinson et al, 2003).

Types of phytoremediation

Phytoextraction: plants remove metals from the soil and concentrate them in the harvestable parts of plants (Kumar et al., 1995; Table 1.1).

Phytodegradation: plants and microbes uptake and metabolize organic contaminants, using enzymes or other metabolites, into less harmful products (i.e. carbon dioxide or water and halide ions) (Burken and Schnoor, 1997).

Rhizofiltration: plant roots absorb metals from running waters (Dushenkov et al., 1995).

Phytovolatilization: volatilization of pollutants into the atmosphere via plants (Burken and Schnoor, 1999; Bañuelos et al., 1997).

Phytomining: extraction of profitable heavy metals from otherwise sub-economic ore bodies to generate a revenue (Robinson et al. 1997a, 1997b),

Phytostabilisation: plants moderate the mobility and bioavailability of pollutants in the environment either by immobilization or by inhibition of migration (Salt et al., 1995; Berti & Cunningham, 2000).

Soils polluted with organics compounds are treated using conventional methods such as soil washing, vapor stripping or thermal desorption, incineration and landfilling. Some organic contaminants can be treated using microbial management. Soils contaminated with metals used to be excavated and landfilled but nowadays acid leaching, physical separation, or electrochemical processes is used. Organic compounds can also be mineralized or degraded by microbes into simpler compounds. On the contrary, inorganic compounds must be immobilized from the ground or physically extracted.

Another alternative are the different types of phytoremediation that can be used for both organic and inorganic compounds. For instance, successful phytodegradation needs organic contaminants to be available for absorption to, or uptake and metabolism by, plant or plant-associated microbial systems.

Table 1.1 Phytoremediation includes the following processes and mechanisms of contaminant removal by Ghosh and Singh (2005).

No.	Process	Mechanism	Contaminant
1.	Rhizofiltration	Rhizosphere accumulation	Organics/Inorganics
2.	Phytostabilisation	Complexation	Inorganics
3.	Phytoextraction	Hyper-accumulation	Inorganics
4.	Phytovolatilization	Volatilisation by leaves	Organics/Inorganics
5.	Phytotransformation	Degradation in plant	Organics

Advantages, implementing and limiting factors for phytoremediation

Phytoremediation provides many advantageous features such as restoration, local decontamination, enhancement and maintenance of the biological activity, physical strengthening of soils, relative cheaper than other options, environmentally friendly and biorecovery of metals (Baker et al., 1991, 1994). Some important observations related to the performance of phytoremediation are:

- It is a non-disturbing technique, with minimal environment damage
- It is solar-energy-driven
- It can be used for a great range of pollutants
- It is much less expensive than conventional remediation techniques
- It may require more than one growing season
- It is species-dependent
- Treatment is restricted to soil as deep as the root system in the plant

- The effectiveness of phytoremediation is dependent on the type of contaminant present in the system.

Based on the phytoremediation concept, the ideal plant accumulator specie must be able to grow quickly, tolerate high concentrations of metals, produce a high amount of biomass and, take up high concentrations of metals from the soil into the roots and translocate these metals to the shoot system. Trees offer many advantages such as: transpiration of large quantities of water; production of large plant biomass; absorption of total dissolved salts within their tissue, long life spans, growth on low-fertility soils that are not productive for annual crops, less frequent irrigation, encouragement of ecosystem diversity, a massive root system that helps soil attachment (Riddell-Black, 1994; Stomp et al., 1993; Bañuelos et al., 1999).

Plants used for phytoremediation

Phytoremediation can be used for almost any inorganic and organic contamination. Approximately 400 plant species from at least 45 families that hyperaccumulate metals have been reported. Several authors have summarized some of the most important hyperaccumulators and heavy metals (Vamerali et al., 2010; Lasat 2002; Adesodun et al., 2010).

Poplar trees (Populus) have been used mainly as biomass or biofuel and ornamental trees or windbreakers to protect agricultural fields from wind erosion. It was until recently that the use of poplar trees have taken the attention due to its physical properties. Some species are used for the clean-up of contaminated soils and groundwater. Hybrid poplars are created to fortify a specific strength from two species. There are more than 35 species of Poplar trees around the world (Figure 1.1). Because of the deep rooting system and its high biomass, poplar trees are perfect for remediation.

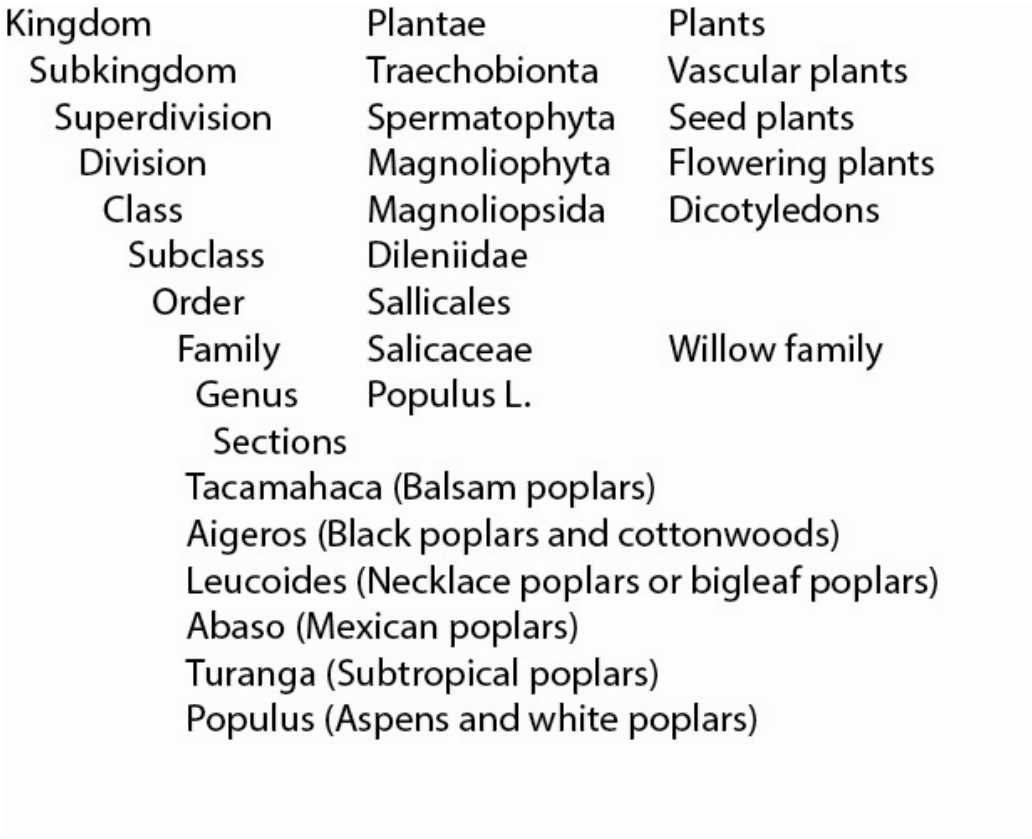


Figure 1.1 Populus taxa

The fossil record first recognize Salicaceae leaves of Populus section Abaso in western North America, from the Palaeocene, around 58 million years ago. These fossils were plants found in riverine habitats. Poplars belong mainly to riparian habitats, but they are also established in drier scrublands and forests (Eckenwalder, 1996). The Salicaceae family contains two main genera, Salix (willows) and Populus (poplars).The taxonomy of the genus Populus has been divided into six sections (Eckenwalder, 1996): Tacamahaca (balsam poplars) and Aigeros, Leucoides, Abaso, Turanga and Populus (aspens and white poplars, Figure 1.2). The Salicaceae are dioecious, and the masculine or feminine flowers are set up in catkins (Rechinger, 1992). Poplars are wind-pollinated species (Eckenwalder, 1996). Poplar trees can grow between 15 to 50 meters in high with trunks up to 2.5 meters width.

Figure 1.2 Poplar tree, Section Populus (P.Tremula)

Salicaceae has developed many characteristics to evolve in flood and disturbance environments. Some characteristics include a large production of seed that are wind spread, a high growth rate of the seedlings, a large root system which anchor the plants and bind unstable substrates and, rapid regeneration (Karrenberg et al., 2002; Stomp et al., 1993).

The aggressive root system of poplars is composed with a downward sinker roots and lateral roots which extend horizontally form the radicle. Lateral and downward roots extend several meters from the base stem. This allows to access beneficial resources over a large area as well as being resistance to erosive forces (Pregitzer and Friend, 1996).

BORON

Boron (B) is discharged into the environment primarily through the weathering of clay-rich sedimentary rocks, household chemicals, geothermal vapor, borax production, leaching from treated wood and paper and, sewage dumping. The presence of boron in soil is restrained in many regions in the world with a high rainfall and seasonal water availability. On the contrary, in the aridlands, groundwater reaches the topsoil by capillary action and vaporizes to leave solutes in soil (Ozturk et al., 2010). Naturally-occurring boron reserves occur in the Cenozoic–Tectonic–Volcanic zone, normally in dry climates around the world (Ozol, 1977; Alonso et al., 1988). It has been estimated that annually 11,800 ton of B are released in coal fly ash from coal combustion (Bertine and Goldberg, 1971).

Boron is a vital micronutrient for plants, thus plays an essential role in some plant functions such as the metabolism of nucleic acids, uptake of Ca^{2+}, root development, flower and fruit formation, cell wall synthesis and structure and, carbohydrates and proteins metabolism, phenol, pollen germination, membrane functionality in higher plants (Eaton, 1955; Marschner 1995; Loomis and Durst, 1992; Gauch, 1972; Abdulnour, 2000; Donghua, 2000 Goldbach et al. 2001; Lou and Yang, 2001).

Residual waters contain several inorganic compounds as well as some toxic compounds. Boron has shown to be a problem due to industrial and agricultural use (Butterwick et al. 1989; Tsadilis, 1997). South Australia, Egypt, Iraq, Jordan, Libya, Morocco, Syria, Turkey (which produces 50% of the world's borax), California, and Chile are areas with boron toxicity problems in agricultural soils (Norman, 1998; Yau et al., 1995; Lyday, 1982). The boron mining leads to a drastic increase in the accumulation of boron in agricultural lands (Parks and Edwards, 2005).

Boron in humans and animals

Boron deficiencies in animals has affected several functions such as bone development, brain roles, macromineral metabolism, energy substrate utilization, immune function and insulin secretion (Hunt and Nielsen, 1981; Hunt et al., 1994; Penland and Eberhardt, 1993; Hegsted et al., 1991; Hunt, 1997; Nielsen, 1997; Bai and Hunt, 1996; Hunt and Herbel, 1992). Boron toxicity in animals usually occurs after levels exceed 100 $\mu g\ g^{-1}$. Boric acid has an acute oral toxicity for mice and rats of about 4000 mg kg^{-1} body wt (Health effects of Boron, 1994). In humans, B deficiency affects the metabolism

of macrominerals, energy, nitrogen, reactive oxygen, and brain functions such as physcomotor performance and response to estrogen ingestion (Nielsen, 1994; Nielsen et al., 1991, 1992). In humans, boron toxicity symptoms include nausea, vomiting, diarrhea, dermatitis and lethargy (Linden et a., 1986). Chronic B toxicity signs are described as poor appetite, nausea, weight loss, and decreased sexual activity (Hunt et al., 1993).

Boron in nature

Boron is found in nature mainly as undissociated boric acid $B(OH)_3$. Plants uptake B from soil in the form of boric acid (Brown and Shelp, 1997). Because boric acid is a non-charged molecule, it is very permeable to the lipid bilayers, thus channel is proportionally dependent on the concentration gradient (Brown and Shelp, 1997, Tanaka and Fujiwara 2008). Boron is required for plants functions in low amounts. Plants take up boron concentrations between 0.5 to 2.0 mg kg^{-1} (Ozturk et al., 2010). In order to translocate to the shoot system of the plant, B requires loading xylem and channelized towards the upwards proportional with the transpiration rate. Ultimately, B is stored in a specific location, usually edges of mature leaves (Brown and Shelp, 1997).

Soils commonly contain 0.5 mg kg^{-1} of boron and lower amounts are considered in the range of boron deficiency for most plant species (Eaton, 1940; Sprague, 1972). The difference between boron deficiency and boron toxicity can be of only 5 mg L^{-1} to many agronomic crops (Nable, et al. 1997; Keren and Bingham 1985). However, significant difference exists between species in their resistance to boron. Boron deficiency symptoms include inhibition of leaf expansion, apical dominance, root elongation, flower development and fruit seed; the meristematic tissue is affected, young leaves become chlorotic and malformation may present (Eaton, 1940). On the other hand, boron toxicity symptoms present in the edges of the old leaves, where those areas become chlorotic or necrotic (Bennett, 1993). This is caused by boron being transported and accumulated at the end of the transpiration stream (Oertli, 1993).

Use of Populus trees for phytoremediation

Populus spp. was first used effectively for managing compounds within residual and inorganic agricultural effluent at Iowa, 1988 (Licht, 1991; Schnoor et al., 1995). Hybrid poplar diversities have a

long lifespan and are perennial, fast growing, and produce a great amount of foliage to harvest (Bañuelos et al., 1999). In addition, the extensive root system permits quick absorption of large volumes of water and soluble ions.

Even though some plant species (i.e. Astragalus, Brassica, spp.) may accumulate much larger concentrations of many macro/micro nutrients than the Populus trees, lower yields compared with poplars would require tremendous high biomass plant yields to reach comparable amounts of B removed by Populus trees (Table 1.2). Additionally, dry matter yields would increment every year and consequently more B should be taken up (Bañuelos et al., 1999).

Poplar trees have been suggested as potential phytoaccumulator for boron (Bañuelos et al., 1997, 2010). By extracting large amounts of water for transpiration and simultaneously taking up B from the soil into their aerial parts, poplars can reduce B leaching from contaminated soils into receiving waters (Robinson et al., 2003).

Robinson et al. (2003) reported leaf concentrations in poplar trees as high as 845 and 1200 mg kg^{-1} DM grown in soils containing boron concentrations between 30 and 40 mg kg^{-1}.

Table 1.2 phytoremediation of metals with Populus spp.

Poplar Species	Heavy metal	Plant Concentration (mg/Kg)	Publication
P. Tremula	Zn	116-3200	Vollenweider et al. (2011); Dos Santos Utmazian et al. (2007)
Populus x euramericana	Zn	130	Di Baccio et al. (2003)
P. euramericana and P. alba pyramidalis	Cd	40-160	Lux et al. (2002)
P. deltoides, P. euramericana and Tremula	Cd	.6 - 13	Pilipovic´ et al. (2005); Vollenweider et al. (2011)
Populus × canadensis, Populus deltoides, Populus×generosa, Populus nigra, Populus alba, Populus trichocarpa	Cd	Roots 9960, leaves 293	Zacchini et al. (2009)
Trichocarpa × nigra, Deltoides × nigra	B	330 - 1200	Banuelos et al. (1999); Robinson et al. (2003)
Trichocarpa × nigra, Deltoides × nigra	Cl	1200 - 6000	Banuelos et al. (2010)

Previous work in Boron phytoremedation

Bañuelos et al. (2010) screened clones from different genetically genomic groups among poplar species to determine their ability to tolerate and survive irrigation with recycled waters high in NaCl salinity and boron under micro-field conditions. They concluded that poplar clones of parentage trichocarpa × nigra, followed by deltoides × nigra demonstrate potential salt and boron tolerance in recycled waters with high salinity and boron concentrations. Finally, they suggested combining field studies with molecular studies to discover the most important factors allowing for tolerant clones that can used to produce predictive molecular markers.

Bañuelos et al. (1999) showed that B is retained with little or no redistribution once B enters the leaf. Consequently, there is a cumulative concentration of B as the leaf ages. Toxicity symptoms manifest the accumulation of B in the leaves. Also, results exhibited that lower leaves positions contained more B than upper leaves positions in each clone, regardless of treatment. Lower and upper leaves contained larger concentrations of B than stem segments in all treatments for all clones. Likewise, salinity increased B concentrations in clones when increasing salinity to $7dSm^{-1}$.

Vollenweider et al. (2011) investigated compartmentation of heavy metal in leaves of populus species. The purpose of their study was the analysis of the heavy metals, macro- and micronutrient distribution in the tree crown and, the micro-localization of heavy metal in leaves from the tissue down to a subcellular level.

ANALYTICAL METHODS

An analytical method with high resolution is required in order to study subcellular ion localization within cells. Energy Dispersive X-Ray Microanalysis is a powerful technique that permits the quantitative analysis of several elements of physiological interest at the subcellular scale (Kevex 1999). Electron microscopy and X-ray microanalysis can describe the elemental constitution in plant cells produced during histochemical or cytochemical assays (Caissard et al., 1992).

Scanning electron microscopy provides an improved higher magnification, depth of field, analytical capabilities, and the benefits of image processing as well as the use and image interpretation from conventional light microscope. The analytical capabilities of the SEM deliver elemental and

crystallographic information that would normally be difficult to obtain using other instruments. The SEM is a powerful and versatile instrument that can find topographic, crystallographic, and chemical information which creates a major tool in research and technology.

Energy-Dispersive X-Ray Microanalysis (EDXA)

Energy dispersive X-Ray microanalysis in a SEM is based on that the electron beam excites atoms to emit X-ray photons which are accumulated by a semiconductor crystal. The energy obtained from the crystal is converted into a voltage pulse proportional to the energy of the X-ray photon. The voltage pulses are arranged by a multichannel analyzer, thus creating the X-ray spectrum (Frey, 2007). The main components of a typical SEM are electron column, scanning system, detector(s), display, vacuum system and electronics controls (Figure 1.3).

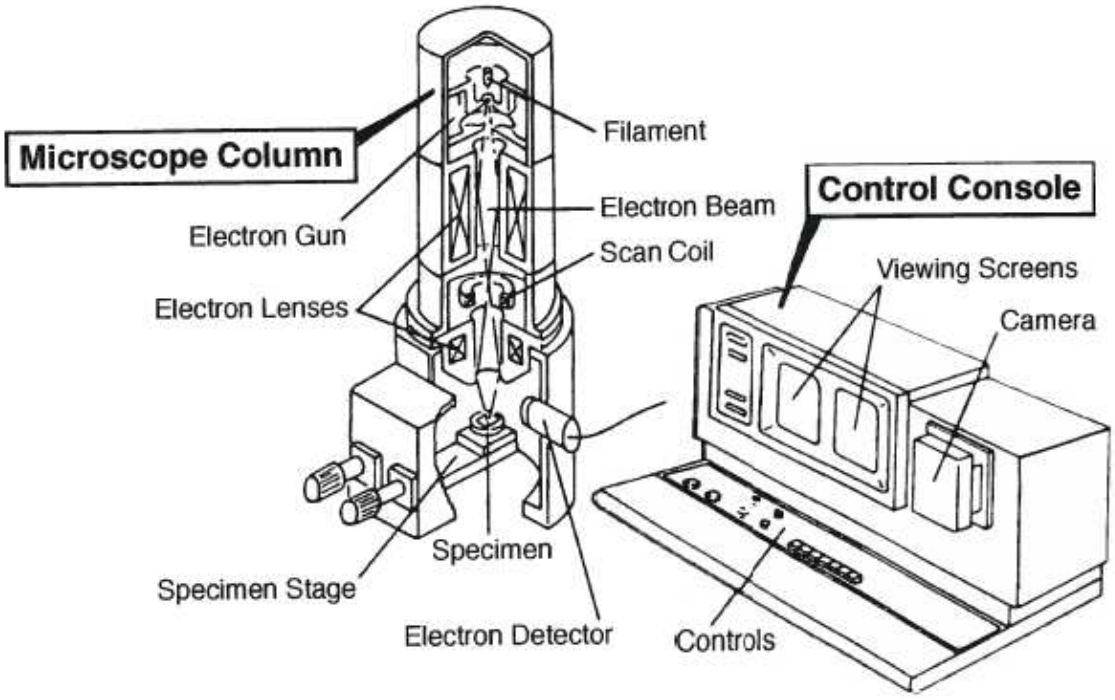


Figure 1.3 Diagram of EDXA. From Goldstein et al. (2003).

Scanning Electron Microscope

The electron column comprises an electron gun and two or more electron lenses that guide the routes of electrons moving down (Figure 1.4). The base of the column goes under vacuum that create a

vacuum of about 1O-4 Pa (Goldstein et al, 2003). The electron gun produces electrons and accelerates them, using an electric field, to obtain kinetic energy in the range 0.1-40 keV (100-40,000 electron volts) (Goldstein et al, 2003). All energy-dispersive spectrometers have a solid-state detector made commonly of a single crystal of Silicon. When an x-ray enters the crystal, these will be absorbed by interaction with an electron of one of the silicon atoms, creating a high energy photoelectron. The expelled photoelectron in time dissipates its energy in interactions (Kevex 1999). The beam begins from the final lens into the specimen compartment, where it interacts with the specimen to a depth of about 1 µm (Goldstein et al, 2003). X-ray photons produce ionization in the detector, causing an electrical charge that is amplified by a preamplifier. Liquid nitrogen is used to cool both detector and preamplifier and minimize electronic noise (intro energy dis). The electronic system of the detector converts the signals to point-by-point intensity fluctuations on the screen to produce an image (Goldstein et al, 2003). The two signals most often used to produce SEM images are secondary electrons (SE) and backscattered electrons (BSE). The quality and resolution of SEM images are function of three major parameters: i) instrument performance, ii) selection of imaging parameters (e.g. operator control), iii) nature of the specimen (CFAMM).

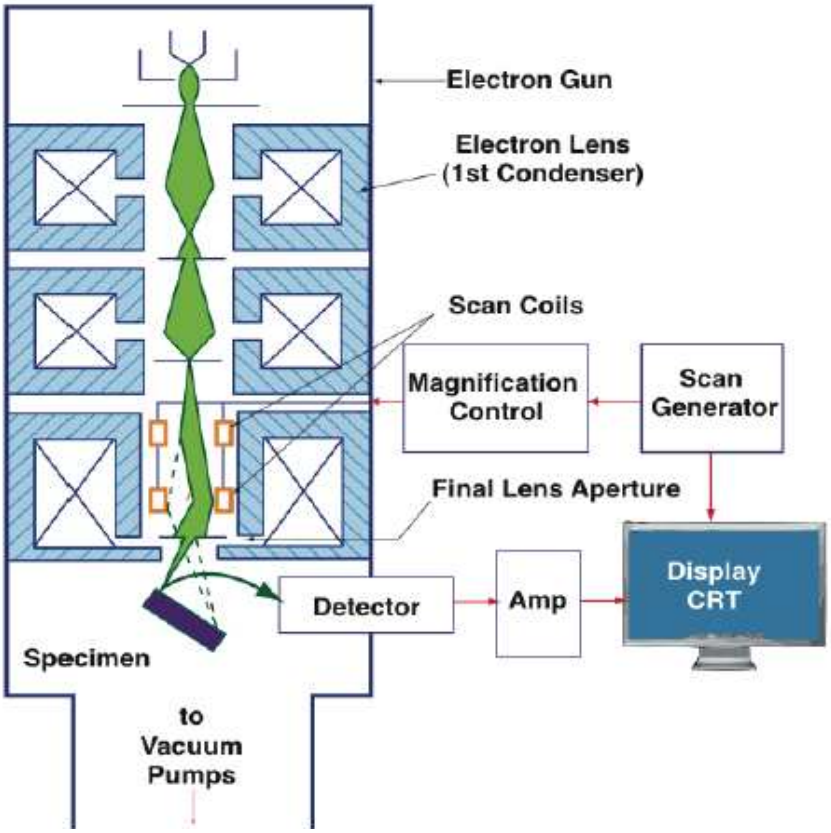


Figure 1.4 Diagram for SEM. From CFAMM (http://micron.ucr.edu/public/manuals/Sem-intro.pdf)

In addition, the voltage pulses are arranged by a multichannel analyzer, thus making the X-ray spectrum (Whallon, 1989). In the energy-to-digital converter, it is measured the height of the voltage pulse from the pulse processor (or the energy of the detected x-ray) and given a channel number. The number of counts in that channel of the multichannel analyzer is then increased by one. The multichannel analyzer controls how the signal information is collected and assembled into a spectrum (EDXA). The larger the number of counts issued by an element, the larger the peaks obtained. In highly automated versions, software programs distinguish the location of spectral peaks, associate them with tabulated energy values, check for discrepancies, and then print out a list of the elements present.

Line segments and mapping

Line segments displays the intensity of a designated X-ray line, element distribution images or 'maps' formed by the SEM. The X-ray spectrum is presented in digitized form with the X-ray energy in

the x-axis and the number of counts per channel in the y-axis. During dot map, the SEM beam at each point on the image is controlled by the x-ray output from the element of interest (Kevex 1999).

Detection limits and resolution

Concentration in the region of 100 ppm the intensity measured on the peak consists mainly of background. The smallest detectable peak may be defined as three times the standard deviation of the background count. Reducing the detection limit requires more counts, which can be obtained by increasing the counting time and /or the beam current. The factors that determine the detection limits in electron and proton x-ray analysis are the counting time, the accelerating voltage, the beam current, the line used to measure the element and the composition of both the sample and the standards. Often the detection limits in the SEM measurements are determined as three standard deviations of the background (CFAM).

Spatial resolution is governed by the penetration and spreading of the electron beam in the specimen. The amount of electron current in the final probe determines the intensity of the secondary and backscattered electron and x-ray signals. Unfortunately, the smaller the electron probe, the lower is the probe current available and the poorer is the visibility of image features. The accelerating voltage (kilovolts) of the beam determines how faithful the image will be in representing the actual surface of the specimen. The operator must control these beam parameters to achieve optimum results in each microscopy mode (CFAM).

Sample preparation and cryopreparation

Since the electron probe analyses only to a shallow depth, specimens should be well polished so that surface roughness does not affect the results. Many samples are electrically non-conducting and a conducting surface coat must be applied to provide a path for the incident electrons to flow to ground. The usual coating material is vacuum-evaporated carbon (~10nm thick), which has a minimal influence on X-ray intensities on account of its low atomic number.

Current preparation techniques for electron microscopic imaging at a sub-cellular level, such as chemical fixation, heavy metal staining and embedding in resin, cause dispersion of disseminated substances and cannot conserve the original functional state of the cells. Cryosectioning of the rapidly

frozen native material represents an alternative method to analyze the plant material with a higher spatial resolution (Frey et al., 2007).

RESEARCH OBJECTIVES

Boron and salt toxicity caused by different sources has affected important agriculture and crops areas around the world. Effective phytomanagement of boron should limit the negative effect of this contaminant on the ground and immediate environment. It is generally defined that plants in phytoremediation employs a strongly enhanced rate of heavy metal uptake, a faster root-to-shoot translocation and a greater ability to detoxify and sequester heavy metals in leaves (Stiles et al., 2010). Nevertheless, except for certain halophytes in which solutes such as sorbitol might inactivate excess B (Rozema, 1996), there is little evidence of data showing detoxification strategies in the ability of tissues to tolerate high B concentrations. It is indispensable to investigate the compartmentalization, microelement localization and tolerance/detoxification mechanisms of contaminants at tree crown and leaf tissues, at a sub-cellular level to identify the strategies and cellular structures in charge of controlling the stress reactions in previously selected hybrid tolerant populus trees.

The aim of this study was to characterize boron and metal contaminants allocation in leaf tissues of selected tolerant vs. sensitive clones combining different histochemical and micro-analytical methods. In order to analyze the distribution of boron and mobile salts and their element environment at tissue and cell level, it was applied qualitative X-ray microanalysis approaches (i.e. point and shot measurements) after cryo-preparation of foliage plant samples. Tolerance to contaminant storage and the eventual structural adaptations will be assessed with histological and cytological analysis using several methods in transmitted light and fluorescence microscopy. Structural analyses will be conducted in Switzerland using facilities at WSL and center for Microscopy and Image Analysis (CMB) of the University of Zurich. The expected results should demonstrate the tolerance mechanisms based on safe contaminant storage and provide insights on the physiological adaptations in tolerant poplar clones.

The outcomes for the boron/salt allocation and plant detoxification mechanisms were:

- It showed why and how the poplar clones tolerate such toxic levels of mineral elements such as B, Na, Cl.
- It identified where the elements are accumulated at cell, tissue and organ level. This knowledge is fundamental also in view of the clone(s) improvement.
- It found data to compare with other allocation/tolerance studies in poplars and other plant material. Also, it will allow to know how efficient are the strategies used by the investigated poplars on a mechanistic point of view.
- It characterized the elements state of the translocated contaminants and may thus identify one of several elements ligands; this knowledge may be bridged with findings by genomic approaches and thus provide guidelines for biotechnical developments
- It provided useful indications on the intoxicated plant material disposal and best harvesting procedures.

Chapter 2: Microelement localization and cellular markers in leaves of Populus spp.

INTRODUCTION

Excessive concentrations of boron (B) are toxic for most plants (Nable et al., 1997). Some effects of B toxicity include reduced growth in shoots and causes chlorosis in leaves (Nable et al., 1997; Reid et al., 2004; Reid and Fitzpatrick, 2009). Translocation of B is insignificant in many plant species and B tends to store in old leaves. Leaf tissues are essential in extracting metals from contaminated soils because the movement of mineral/elements in plants is determined by leaf transpiration (Marschner, 1995). Understanding the process of how the leaves allocate and detoxicate metal contamination may provide evidence to infer tolerance mechanisms of vascular plant to metal stress.

Irrigation water quality and drainage water disposal, in the western San Joaquin Valley of Central California, have been significant for irrigated agriculture, after inorganic salt contaminants, particularly boron and other soluble salts accumulated at Kesterson National Wildlife Refuge (Ohlendorf et al., 1986). Evidence suggests that recycling saline water originating from agricultural drainage or from shallow ground waters is a desirable site for disposing of the saline water (Oster, 1994). Recycling of residual waters for irrigation could have application for more than 250,000 acres of drainage-impacted soils of the Western San Joaquin Valley, even though long-term use needs a serious evaluation that includes irrigation and irrigation delivery systems (Dudley et al., 2008), and salt management. A practical water reuse strategy in Central California would require the selection of salt and boron tolerant crops and trees for use with waters high in salinity (e.g., 10 dS/m) and boron (10 mg/L) (Lin et al., 2002).

Tissue and cellular allocation of Boron in plants

Comprehending the process to reduce toxicity is a current interest because it offers the means of manipulating metal tolerance to develop crops for phytoremediation purposes, especially highly contaminated soils (Salt et el., 1998). Characterization of boron distribution in shoot tissues of higher plants is limited to a few, mostly herbaceous, plant species. Little is known of how the plant tissues can manage such high toxicity B levels at a subcellular level (Reid and Patrick, 2009).

G. arrostil. and P. distans are known B hyperaccumulators with concentrations that reached ~2400 and ~6000 mg B kg^{-1} DW, in the roots and shoot tissues, respectively (Stiles et al, 2010). Recently, Ture and Bell (2004) analyzed several species that have grown in soils containing high levels of boron. At the end of the study, 38 families belonging to 88 taxa (57 species, 20 subspecies, and 11 varieties) were determined. Two plant taxa were found with the highest concentrations Catapodium rigidum (L.) and Gypsophylla perfoliata L.

Stiles et al. (2010) have offer three hypotheses of how tolerant B species may respond to high concentrations of toxicity.

1) The plant is capable to restrict the accumulation of B to above-ground biomass (Hayes and Reid, 2004);

(2) The plant restricts the distribution of B from roots to shoots when supply concentrations reach >100 mg B/L. Then, it loses its ability to restrict translocation of B from the root to shoot;

(3) The plant is able to tolerate high concentrations of B in both root and shoot tissues. After 1250 mg B L^{-1}, the plant behaves as hyperaccumulator to handle the high toxicity concentrations.

So far, these hypotheses has been presented in boron tolerant species but little information is found regarding the toxicity mechanisms in the leaf tissues and how such high levels of metal are tolerated.

Stress and tolerance structures

Various studies have identified the cell wall as the main deposit structure for B for different plant species (Dannel et al. 1998; Kobayashi et al. 1997; Hu and Brown 1994; Martini and Thellier 1993). Dannel et al. (2001) presents a review with the most prominent results in Boron absorption from the soil and translocation from roots to shoots and storage in plant tissues.

Boric acid is distributed from roots to leaves via xylem vessels (Raven, 1980). Via the petioles, the xylem stream moves into the leaf where it is mainly transported in the veins to sites of fast evaporation, such as leaf tips or leaf margins (Sattelmacher 2000).

The water insoluble B bonds to pectic polysaccharides especially rhamnogalacturonan II (RG-II). Yamauchi et al. (1986) were the first to present the likelihood of cell wall polysaccharides which contain

B. The majority of cell wall B is present as a B-RG-II complex, with RG-II being the selected carrier of B (Matoh et al., 1996).

It was proposed that pectin is involved in the induction of plant defense responses based on the capacity of oligogalacturonides from plant cell walls or pectins (Spiro et al., 1993) to origin the production of phytoalexins (Nothnagel et al., 1983), and reactive oxygen species (Ridley et al., 2001). Thickened and pectin-rich cell walls (Bowes, 1997), might be thus a significant B sink.

Some authors have corroborated that flavonoids and other phenolics are used as strong antioxidants for plants, thus improving stress tolerance (Rice-Evans et al. 1997; Barbehenn et al., 2006; Hagerman et al,. 1998; Mellway and Contable, 2009). Additionally, a higher accumulation of phenolic compounds (especially condensed non-soluble tannins) was detected in leaves of P. euphratica (salt tolerant) than in those of P. canescens Sm. (salt sensitive) (Janz et al. 2010). In another study, increases in antioxidative complexes were correlated to increases in polyphenols caused by chemical effectors that resulted in ameliorative results on stressed plants (Muthukumarasamy et al., 2000). Phenolics compounds can prevent negative effects of oxidative stress by recovering free radicals, thus, preserving cell and tissue integrity (Janz et al. 2010). Polyfunctional proanthocyanidins are good chelators that can form stable complexes with many metal ions (Kennedy and Powell, 1985; Okuda et al., 1982; Slabbert, 1991; Powell and Rate, 1987). It can be concluded that more polyphenols produced indicate that more antioxidants are available to ameliorate stress effects.

Depending where metals are accumulated at tissue and cell level, they can generate either little (Cosio et al. 2006) or a sequence of oxidative stress reactions leading to hypersensitive-like responses (Vollenweider et al. 2003; Martin et al. 2006).

This chapter aimed to examine the B allocation at the tissue and cell levels in the leaves, and to detect the associated structural changes and to characterize the main plant responses. Different histological approaches in light microscopy were combined and changes in structural composition within cells and leaf blade were analyzed. Stress tolerance mechanisms and cellular markers are pointed and classified according to physiological response.

MATERIALS AND METHODS

Three of the top Populus clones rated as "Good" performers during microfield studies conducted by Banuelos et al. (2010) were selected to grow under adverse field soil conditions because they were repeatedly the heartiest clones during the first selection process. These clones are: 13-366 (designated as RRR Yellow, tolerant), 345-1 (designated as RRR Red, intermediate), and 347-14 (designated as RRR Blue, sensitive). In spring of 2006, 80 cuttings were taken respectively from the parents of these selected three clones, and planted at Red Rock Ranch in the Western San Joaquin Valley in Central California at an altitude of 80 m above sea level. Each clone was planted 3 m apart and replicated four times within one block; there were a total of 20 blocks running north and south. The environment at Red Rock Ranch is very dry, hot, and exposed to high light intensity. The average summer (June – August) temperature at Red Rock Ranch ranges from a high of approximately 35 degrees C during the day to a low of approximately 16 degrees C at night. The average winter temperature (January and February) at Red Rock Ranch ranges from a high of approximately 12 degrees C during the day to a low of approximately 3 degrees C at night. The relative humidity ranges from approximately 28% to approximately 70% in the summer (June – August), and approximately 70% to approximately 95% in the winter (January and February). Horizontal solar radiation averages approximately 8 kWh/m^2/d in July with an annual total ranging from approximately 1.8 MWh/m2 to approximately 2.0 MWh/m^2. There is virtually no rainfall for four months from May through September, and evapotranspiration (ETo) remains high with daily rates of approximately 7 mm (7 L/m^2/d).

Red Rock Ranch has clay soils, containing high concentrations of salt (Na_2SO_4, NaCl, $CaCl_2$, Na_2SeO_4, $CaSO_4$, $Na_2B_4O_5(OH)_4$, and $CaB_3O_4(OH)_3$) and boron. The Red Rock Ranch soil composition is classified as an Oxalis silty clay loam (fine montmorillonitic, thermic Pachic Haploxeral with a well-developed salinity profile. Soil salinity varies from approximately 4 dS/m to approximately 8 dS/m, while soluble boron varies from approximately 4 mg/L to approximately 7 mg/L. The presence of poor quality shallow groundwater underneath the trees (fluctuates between approximately 1 m to approximately 3 m from soil surface) contributed to additional salt and boron stress for the trees. Periodic groundwater sampling showed that ECe and boron ranged from 10 dS/m to 18 dS/m and 10 mg/L to 18 mg/L, respectively.

Two locations were used as treatment location designated as: Diener and Parlier. Diener location (Five points, CA) was irrigated under field conditions (multiple element contamination). Parlier location (Parlier, CA) receives normal irrigation without any treatment and it was used as control. Sampling design consists of three clones (three replicates for location) with two locations at two different branch positions (high and low) from two clusters (11-2-4, sub-lethal and 1-1-1 thriving) of a total of 24 samples.

After 6 years of growth under these adverse conditions (described above), the leaf samples were collected by Dr. P. Wollenweider. Samples were harvested as branch samples up to 30-35 inches. They were processed for high pressure freezing (HPF) within 48 hours and by this time showed more evidence of injury due to storage.

Additional leaf sample aided out for drying and subsequent element analysis on the 01.10.10, thus 4 days after sampling. These leaves included the 3 used for microscopy sampling +2 closest neighbors, 3 just further were sampled in view of eventual boron analysis once in Zurich.

Samples stored in 1-hexadecene, 20 °C up to 6.5 hours prior to infiltration by evacuation (N550 mm Hg) and HPF using and HPM 010 machine (pressure: 600 mili-second, -157 °C; 2-5") using a 200 µm deep, gauged platelets (leaf blades thickness estimated at about 120 µm). Sample loaded on platelet in 1-hexadecene and transferred to the specimen holder.

Sampling at leaf level was performed at the mid rib on leaf. Each time, 20.8 mm disks were sampled. There were 2 types of preparation vein and leaf blade. Each sample corresponds to a group of 3 leaves. Two branch positions were sampled, in principle; at shoot basis and at shoot top (by the tissue of sampling (the growth was completed and the buds formed).

Histochemical Analyses

The experiments were performed in the facility of the Swiss Federal Institute of Forest, Snow and Landscape Research (WSL) at Birmensdorf, Switzerland. For the microscopical analyses, leaf disks were sampled either in the middle portion or in the apex and the base of one leaf at two different shoot positions, according to the visible symptoms.

Leaf material was sampled from the middle leaf portion of an older leaf on one plant per treatment. Polysaccharides (starch and sugars), cellulose, pectin and phenolic compounds were observed in 1 metachromatic and three stained sections. The histological and cytological structure was further analyzed with semi-thin sections. Sections 2.5 µm thick were cut with a Reichert ultramicrotome, stained and mounted in DePex for light microscopy and fluorescence microscopy. Five cuttings from three clones, two leaves and two different leaf positions per treatment were examined each time.

Sample sections were observed using a Leica microscope Leitz DM/RB, 5x to 100x objectives and diascopic light illumination. Observation by fluorescence microscopy (epifluorescence filter combination: excitation band pass filter 340-380 nm, emission long-pass filter 425 nm). Micrographs were taken using the digital Leica DC500 camera interfaced by the Leica DC500 TWAIN software embedded in the Image Access Enterprise 5 (Imagic, Glattbrugg, Switzerland) image management system. The histochemical and histological markers were selected so as to indicate the kind of physiological response and its localization in the leaf, as a consequence of B stress.

Statistics

Statistical analysis was performed using the ANOVA procedure of SPSS version 20.0 (SPSS Inc., Chicago IL) and a pairwise comparison procedure (Tukey test) for significant differences. For the leaf samples, a 2-way factorial ANOVA was conducted using location, leaf position and clone as independent factors. All data in figures and tables represent means of replicates ± standard error.

RESULTS

Morphology and biomass

Physical differences between the two locations are clear noticeable. Tree height was slightly affected by the application of the multiple contamination (Figure 2.1). In contrast, biomass was statistically significantly for the location, clone and the interaction location and clone (Table 2.1). The clone 13-366 showed a higher (138.7 cm^2) biomass increase followed by clone 345-1 and clone 347-14, respectively (Figure 2.2).

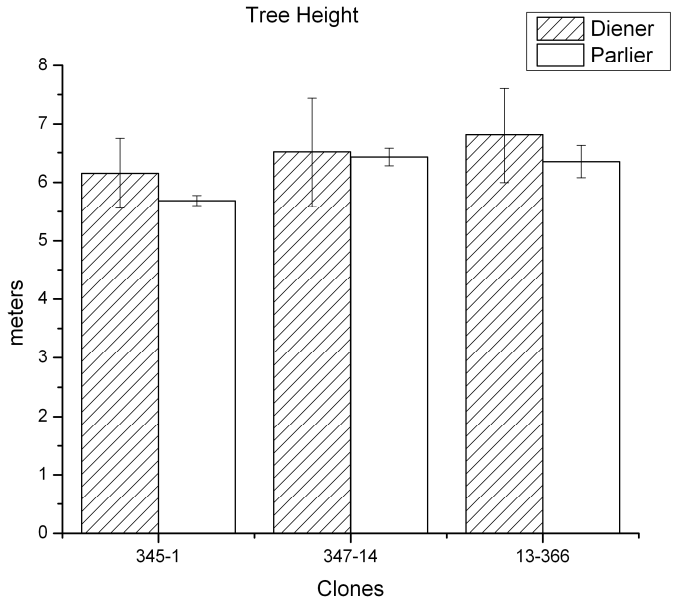


Figure 2.1 Tree heights for the three different clones at the two locations. All values represent means ± SE.

Table 2.1 Basal Area from three Poplar clones

	Basal Area (cm^2)	
	Diener	Control
345-1	76.03 (± 1.78)[a]	8.86 (± 17.2)[a]
347-1	51.23 (± 1.32)[b]	8.54 (± 15.4)[a]
13-366	138.7 (± 0.98)[c]	11.67 (± 22.9)[a]

All values represent means ± SE of four replicates (N=6)
Small letters indicate significant differences between locations (Tukey HSD, $p<0.05$)

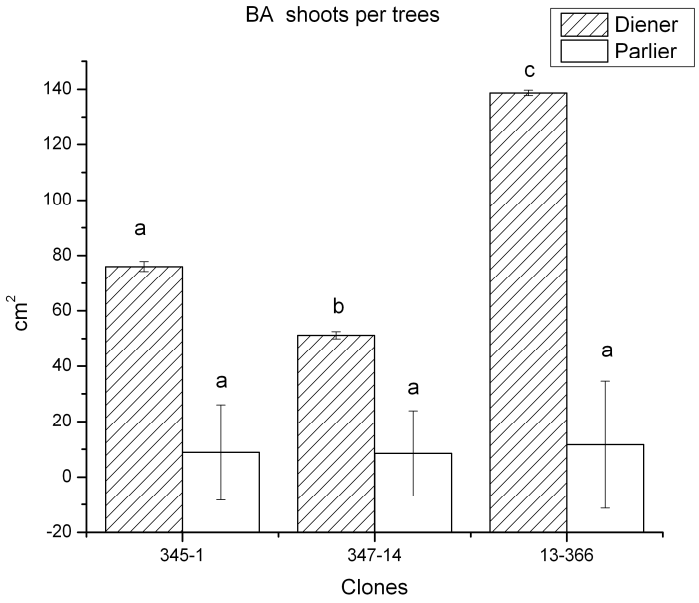


Figure 2.2 Basal area for the three different clones at the two locations. All values represent means ± SE. Small letters indicate significant differences

The symptoms were most pronounced in leaves near the shoot base (data not shown). Necrosis percent is apparent in treated clones especially in the cluster thriving cluster (Table 2.2). Necrosis was statistically significant in cluster and leaf branch position. No necrosis was observed in Parlier location. Visual effects detect necrosis in leaf tips and margins. This is in agreement with other studies (Rees et al., 2011).

Table 2.2 Percent necrosis per cluster

	Cluster	
	11-2-4	1-1-1
% Necrosis	7.92 (± 1.82)	28.3 (± 5.9)
Range %	2.5-15	5-45

All values represent means ± SE of six replicates (N=6)
Necrosis is only observed in treated samples
$p<0.001$

Physical leaf measurements (weight and area) were used to calculate the leaf mass per area (LMA). Higher LMA values were estimated at Diener than in Parlier location (Table 2.3). LMA was statistically significant in the location (Diener vs Parlier) and the interaction location and leaf branch position. Leaf branch position was higher closer to the main stem (leaf position 1 vs leaf position 2). For

comparison, decidous trees median value (75 g m^2) for different species was reported (Poorter et al., 2009).

Table 2.3 Biomass: Leaf mass per area on different leaf positions

	Leaf mass per area in gr m^2	
	Diener	Parlier
Leaf Position 1	111.4 (± 3.0)	79.6 (± 4.8)
Leaf Position 2	108.8 (± 17.0)	98.5 (± 2.6)
Decidous spp*		75

All values represent means ± SE of six replicates (N=6)
No significant difference between means
* Decidous trees median value (N=420). From Poorter et al. 2009

All the elements of interest (B, Na, Cl) are positively correlated (Table 2.4). Boron and sodium are positively correlated to branch height, distance of branch from base, necrosis percentage, leaf mass per area, average basal area and negatively correlated to the number of shoots per tree. Chlorine is only negatively correlated to tree height. Bañuelos et al. (2010) found a positive correlation between the increase in salinity and the decrease in poplar growth. The effects of B and sodium are clearly interconnected. Interestingly, there is no effect of B and Na in the three heights. Additionally, the increase of B-Na levels intensifies the necrosis observed and the biomass is increased. In contrast, the increase of B-Na levels reduces the number of shoots per tree and their distance to the branch basis but increase the branch height.

In addition, chlorine is also positively correlated to Cu and negatively correlated to Ca and P. Boron and Sodium have the same positive (Cl, Ag, As, Cd, Co, Se and Zn) and negative (Fe, K, Mg, Ni and Pb) correlation (Figure 2.4).

The excessive levels of boron and sodium showed the same effects on the plant and in the trends with other elements. This provides evidence to establish the mutual connection between this two elements and their possible effects on poplar trees.

Table 2.4 Correlation between several factors and element concentrations

	Branch height (cm)	Distance to branch basis (cm)	% Leaf necrosis	Leaf mass per area (gr per cm2)	Deposits per tissue (um per mm2)	Tree height	Numb shoots per tree	Avg BA per tree	Total BA per tree	Cl	B
Distance to branch basis (cm)	-.706**										
% Leaf necrosis	.732**	-.902**									
Leaf mass per area (gr per cm2)	.781**	-.522**	.693**								
Deposits per tissue (um per mm2)	-	-	-	-							
Tree height	-.768**	.756**	-.784**	-.606**	-						
Numb shoots per tree	.767**	-.791**	.757**	.686**	-	-					
Avg BA per tree	-	-	.506*	-	-	-	-.799**				
Total BA per tree	-	-	-	.492*	-	.597**	-	.490**			
Cl	-	-	-	-	-	-.453*	-	-	-		
B	.658**	-.785**	.899**	.639**	-	-	-.730**	.643**	-	.437*	
Na	.659**	-.911**	.913**	.506*	-	-	-.799**	.726**	-	.547**	.883**

Significance levels are indicated by asterisks: *p≤0.05; **p≤0.01; - = non-significant.

Cl	↑	B Cu Na
	↓	Ca P
B	↑	Cl Ag As Cd Co Na Se Zn
	↓	Fe K Mg Ni Pb
Na	↑	Cl Ag As B Cd Co Se Zn
	↓	Fe K Mg Ni Pb
↑		Positively correlated
↓		Negatively correlated

Changes in leaf structure

The most typical changes in Populus tree leaves in response to the metal contamination occurred in the lower leaf blade. The cell wall chemistry was modified, especially in the outer cell wall layer (Figure 2.3A versus Figure 2.3B). Changes in palisade and spongy cells observed by light microscopy (Figures 2.3A versus 2.3B) included progressive cell wall thickening, condensation of the nuclei-chloroplasts (Figure 2.3E) and occasional accumulation of vacuolar phenolics. Cell content is typical of low activity cell with a few organelles, thin cytoplasm and, huge vacuole (Figure 2.3 D). Plastids are probably in the form of leucoplast (containing no starch, differently of spongy parenchyma cells). Cell walls structures are similar to that in the lower epidermis. Cells walls are thickened with at least a pectin layer and a cellulose-rich inner layer. The area more affected in all the clones was the lower mesophyll. The spongy parenchyma cells presented tannins accumulation as well as pectinic wart-like droplets in some cells (Figure 2.3D vs Figure 2.3C). Lower epidermis showed squeezed cytorhisis at some places (not shown).

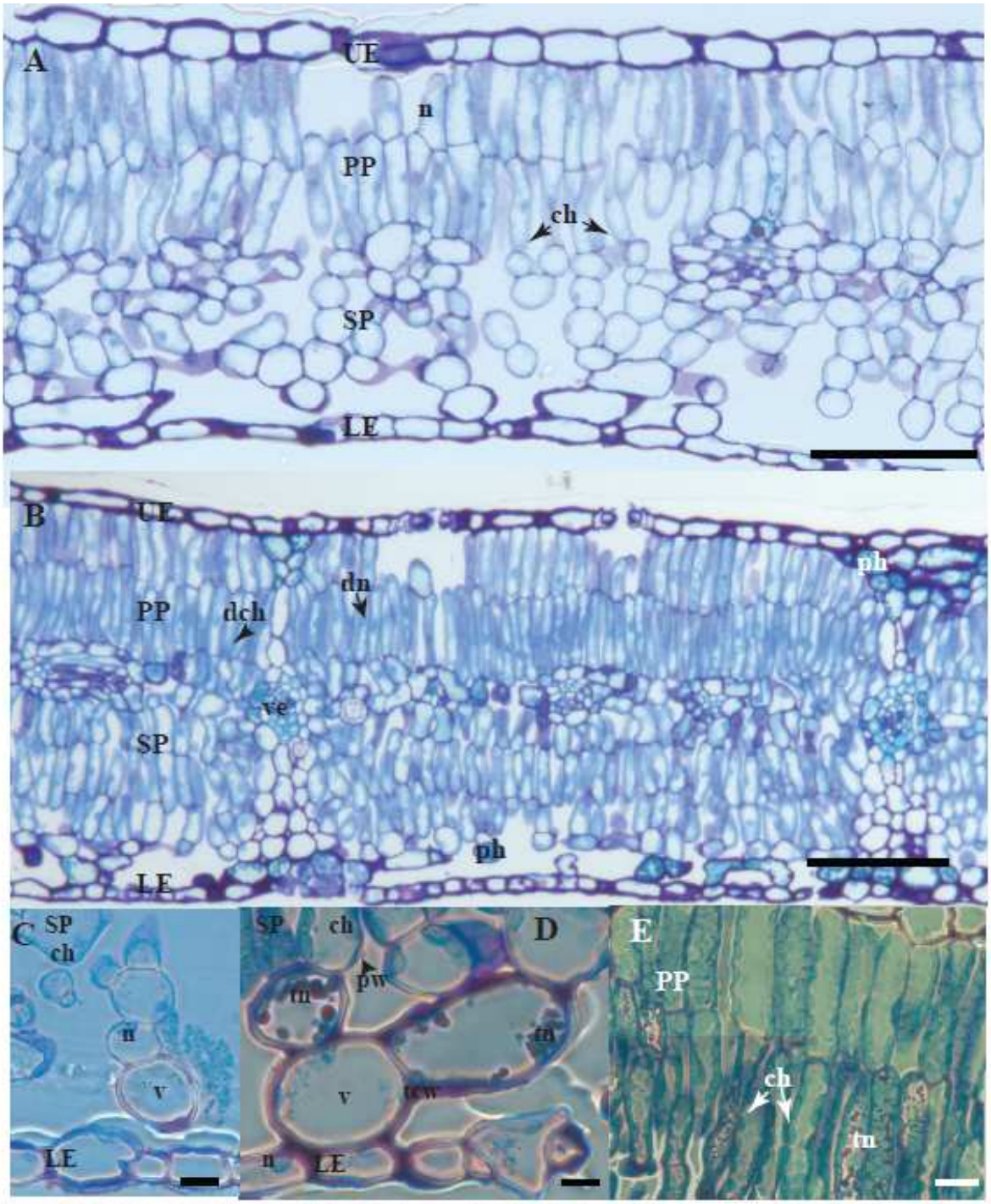


Figure 2.3 Histological changes in Populus trees on leaf blade tissue. Tissues showed injuries of varying degrees for the different leaf structures. The greatest effects manifest in the lower mesophyll in the lower epidermis (LE) and spongy parenchyma (SP) with a clear thickening of the cell walls (tcw). Palisade parenchyma (PP) showed a more uniform structural changes than spongy parenchyma with dense chloroplasts (dch), dense nuclei (dn) phenolic accumulation on both and sometimes including pectinic wart-like droplets (pw). The lower epidermis was more affected than the upper epidermis (UE).Vacuoles (v) accumulated phenolic material (ph). Effect in the lower blade epidermis are shown in C-E. In control leaves (C), no cell wall thickening is present. In Diener leaves (D), is observed tannin accumulation (tn, red spots), thickening of cell walls and large vacuoles. (E) Palisade parenchyma in contaminated leaves showed condensed chloropast (ch) with tannins. Abbreviations: ve, veinlet; ch, chloroplast; n, nucleus. Control: A,C; Diener: B, D. Bars: 100 um (A,B), 10 um (C, D), 20 um (E). Staining was with toluidine blue and p-phenylenediamine (A-D), and viewed with phase contrast microscopy (C, D, E).

Chloroplast accumulation on Parlier leaves showed to be higher than the Diener leaves (Figure 2.4A vs 2.4D). Pectin-rich outer layer on the cell walls is thickened in comparison with the control cells (Figure 2.4A vs 2.4D). Oligomeric proanthocyanidins (=condensed tannins) were also deposited at the same cell wall locations (Figure 2.4A and Figure 2.4E). Starch polysaccharides were accumulated in higher magnitude in Diener leaves than in control leaves (Figure 2.4B vs Figure 2.4E). The inner cellulose-rich layer did not show any change for both locations (Figure 2.4C vs Figure 2.4F). This may indicate no reaction to accumulation in contrast to pectin layer.

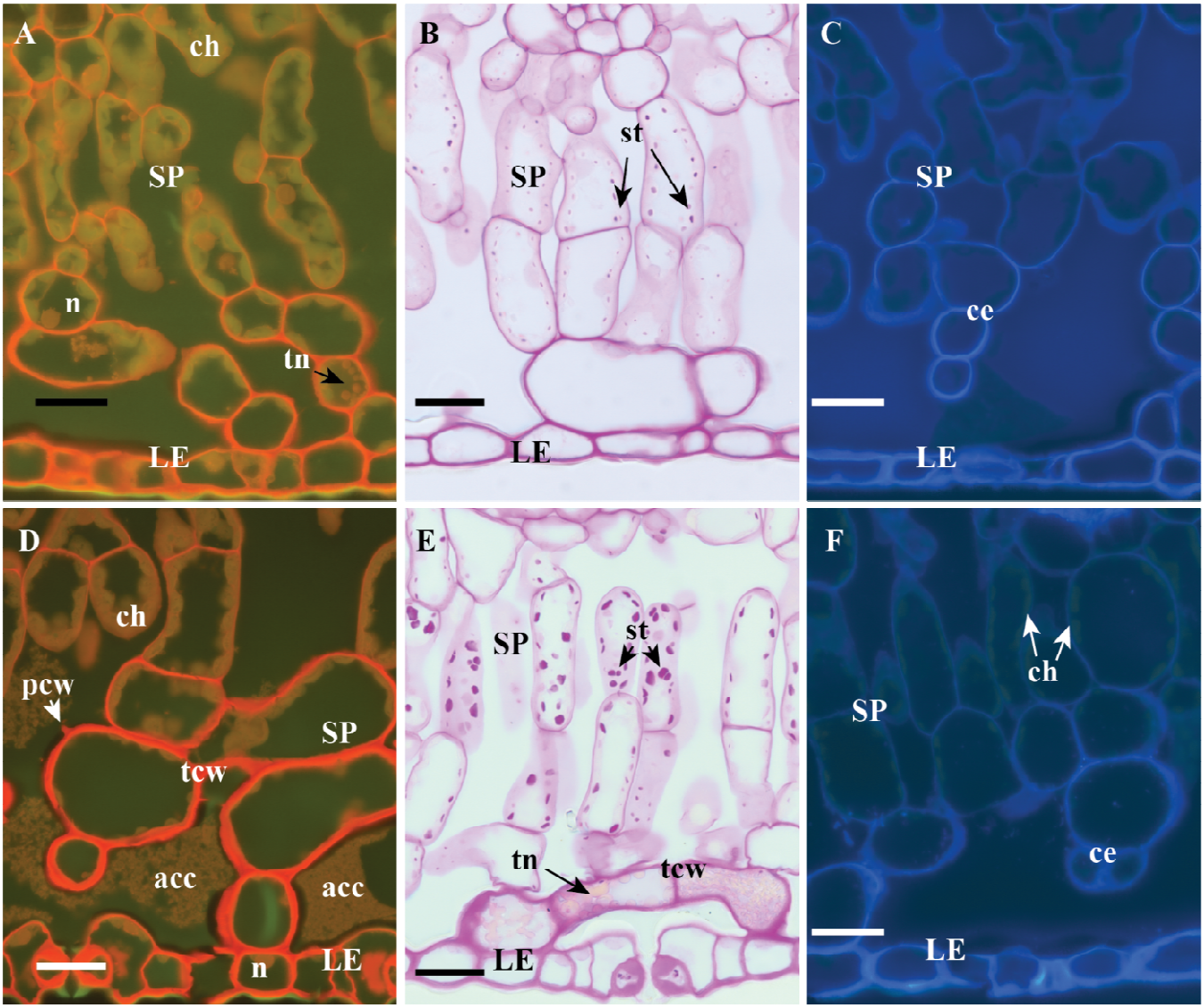


Figure 2.4 Structural changes in the structure of assimilative cells inside the leaf mesophyll of Populus trees induced by metal contamination. Control (A) cells contain vast amount of chloroplast (ch) and some cells present tannins (tn). In contrast, cells in contaminated samples (D) showed intercellular abiotic deposits (acc), thickening of pectin-rich outer cell cell-wall layers, and some cell present pectinic wart-like droplets (pcw). Control cells (B) showed polysaccharides (eg. starch) distribution in the spongy parenchyma (SP). Contaminated cells (E) exhibit thickening in the cell walls on both spongy parenchyma and lower epidermis (LE), with the presence of tannins and higher accumulation of starch (st). Cells from control location and contaminated (C vs F) showed same thickness of the cellulose-rich inner layer in the cell wall (ce). Revelation method: Coriphosphine (A, D), Calcoflour (B, E) and PAS (C, F). Abbreviations: nucleus (n). Bars: 20 μm (A-F).

Intercellular abiotic deposits in the form of granular material accumulated in the lower leaf blade apoplast (Figure 2.4A, 2.4B). It is located on a water/mineral element route and is associated to local cell wall thickenings and wart-like protrusions - both are typical stress reactions (Helmer et al., 2007). The interccellular deposits were observed on both locations for most of the clones. This may be an indication of the normal efflux mechanism in poplar trees. In order to understand better the nature of the intercellular deposits, it was measured the number of deposits per length tissue (Figure 2.6). The value for Diener location is higher than in the control location. This indicates the relationship between the deposits and the contamination site.

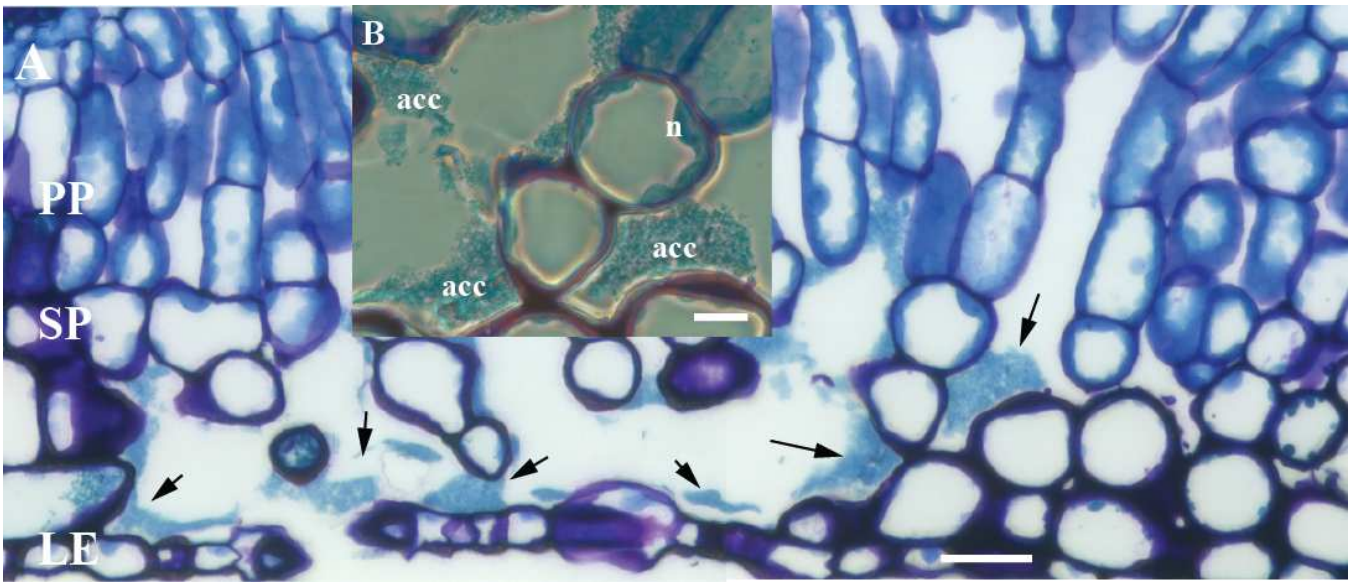


Figure 2.5 Intercellular abiotic deposits. (A) The lower mesophyll intercellular space showed granular material (B, indicated with arrows) accumulated in the apoplast. Samples were stained with toluidine blue and p-phenylenediamine (A), and viewed with phase contrast microscopy (B). Abbreviations: lower epidermis (LE), spongy parenchyma (SP), n (nucleus), accumulation deposits (acc). Bars: A 25 µm, B 10 µm.

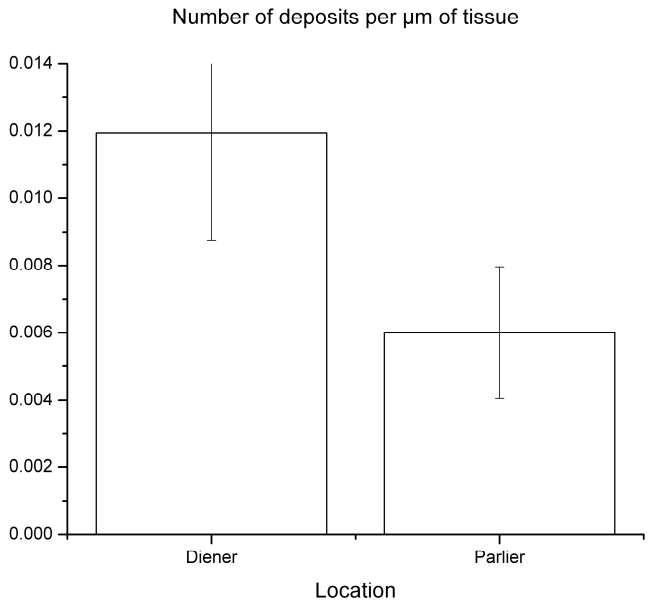


Figure 2.6 Intercellular number of deposits relationship with location. Statistical significant difference $p < 0.05$

Table 2.5 Physiological response and cellular markers in poplar leaf tissues.

Physiological response	Observed cellular markers	Figurers	References
B storage	a)Cell wall thickening	Fig. 2B, 2D, 3D	a) Cosio et al. 2006; Hermle et al. 2007
	b) Proanthocyanidins	Fig. 2D, 2E, 3A, 3	b) McDonald et al., 1996; Kennedy and Powell, 1985; Okuda et al., 1982; Slabbert, 1991; Powell and Rate, 1987
	c) Pectin	Fig. 3D, 3E	c) Yamauchi et al., 1986; Matoh et al., 1993;
	d) Phenol compounds	Fig. 2B	d) Janz et al. 2010; Rice-Evans et al. 1997; Barbehenn et al., 2006; Hagerman et al,. 1998; Mellway and Contable, 2009
	e) Intercellular abiotic deposit	Fig. 3D, 4A	
	f) Pectinic wart-like droplet	Fig. 2D, 3D	f) Hermle et al. 2007

DISCUSSION

Biomass and morphology

Poplars on a tree level have showed the strategy to substitute rather than to repair its injured leaves (Günthardt-Goerg and Vollenweider et al., 2003). Statistical analyses show little differences between clones even though the three clones have shown significant differences in other studies

(Bañuelos et al., 1999, 2010). It is ascertained the differences in the biomass but not in the tree height regarding the metal contamination. Trees, on a pluri-annual basis, suffered from the multiple contamination despite their tolerance. A recent study has shown this correlation (Rees et al., 2011). Most visible symptoms in leaves are segregated to tip and margins. Symptoms were generally more apparent in leaf apexes and in younger leaves than in other leaf parts. Leaf branch position closer to the main stem showed the higher necrosis percent in leaves.

Intercellular deposit

Observed deposits of abiotic origin, various cell wall thickening and, symplastic defense reactions were seen with little difference between the Diener and Parlier leaves. The thick cell walls and the rather insensitive ongoing cell physiology processes therein might contribute to the leaf tolerance to salt stress, given the water and mineral element fluxes occurring in the lower leaf blade tissues

Histochemistry

Characteristic changes in the structure of cells and cell walls were associated with increases in metal exposure. Besides injury to different organelles, several markers indicated specific adaptations of the cell physiology to B storage (Table 2.5).

Condensed tannins were apparently involved in metal detoxification. Visible injured spots in the leaves of poplar trees resulted from distinct groups of necrotic cells in the assimilative tissue bounded by degenerating cells filled with phenolic compounds including proanthocyanidines. They can thus complex different metals with more or less efficiency (Okuda et al., 1982; Slabbert, 1991; Powell and Rate, 1987). Antioxidant functions of proanthocyanidins could also mitigate the oxidative stress caused by metal contamination (Janz et al. 2010; Rice-Evans et al. 1997). Pectin was certainly a main B binding in pectin-rich sites.

Contaminant distribution at leaf level appears to accumulate primarily in epidermis and lower leaf blade tissues. In this latter location, they appear to relate to significantly increased amorphous deposits in the intercellular space. The majority of cell B is associated with pectins within the cell wall.

The localization of B in the cell wall, its association with cell wall pectins, and the contingent effects of B on cell wall extensibility suggest that B plays a critical, although poorly defined, role in the

cell wall structure of higher plants. The localization of B in plant cell walls may indicate that B has a structural role in the cell wall matrix or, alternatively, that B is required for the synthesis of new cell wall material.

Boron accumulates along the mineral nutrient pathway, especially close to its end point where water is then evapotranspirated. The tolerance mechanism appears to be a coincidence of a naturally resistant tissue on the pathway of an overloaded nutrient flux. At the principal accumulation site of contaminants in the lower leaf blade tissues, there is a 2-3 cell layer-thick hypodermis, a structure rarely observed in poplar foliage and non-xerophyte species. This tissue has thickened cell walls, a good allocation site for boron, and these cell walls can be further thickened in response to contaminant accumulation. Hypodermis is a specialized tissue, with low cell physiological activity, and, being not involved in vital processes such as photosynthesis and assimilates transport, is an ideal site for B and salinity detoxification. This hypodermis structure was also observed in the three clones from Parlier, and thus appear to be constitutive in foliage of these clones.

In conclusion, tolerance stress observations remarks: 1) All the symptoms that poplar clones presented are indicative of extra cellular oxidative stress, 2) there are relatively few changes in the cell structures between control and contaminated samples, 3) the only reaction seen is the thickening of cell wall. B-salt storage in the intercellular space rather than in tissues demonstrated to be a safer allocation mechanism. In general, stress and tolerance reactions indicate that cells in the hypodermis structure are well suited for accumulation.

Chapter 3: Element localization in leaves of Populus spp.

Introduction

Numerous metals in contaminated soils are stored in plants that can disturb plant metabolism (Siedlecka, 1995), translocation to shoots (Marschner, 1995) and accumulation within the many cell and tissue compartments (Verkleij and Schat, 1990; Ernst, 2006). Several tree species can grow at places with moderate to fairly large contamination (Watmough and Dickinson, 1995; Lepp and Madejon, 2007). They also been proposed for phytoremediation purposes (Robinson et al., 2003; 2007), implementing their rapid growth and large biomass (Laureysens et al., 2004, 2005). It is indispensable to provide evidence in order to understand metal uptake in trees and at the same time, implement novel applications aiming tree tolerance mechanisms.

Some regions of Central California have identified, in saline residual waters, moderate to high levels of unusual naturally occurring boron (B). Boric acid (as water-soluble B) concentrations of 5 mg B L^{-1} in soils and irrigation water can be lethal for numerous agricultural crops (Keren and Bingham, 1985; Maas 1987) as it can be easily absorbed by the plant.

The tolerance and accumulation of boron in plants is argued to be mainly by sequestration in the cell walls, however, some species accumulate B in different compartments in cell and tissues (Table 3.1). Dannel et al (1999) suggested that after increasing the external B supply by a factor of 16,000 in sunflower plants, cell walls in roots and leaves also increase by a factor of 2.8 and 22, respectively. They argued that cell walls behave as chemical absorber where B binds and cannot be easily mobilized or detached. Even at low B supply, significant amounts of B were found in the symplasm of roots and leaves, although empty binding sites were available in the cell wall (Dannel et al., 1998). This suggests that B found in the symplasm plays an important function in the plant metabolism. Kobayashi et al (1997) established that around 90% of B is bound to the cell wall in cultured tobacco cells. Several authors have proved that the majority of cellular B is present in cell wall and there is an exclusion of B from the symplasm (Matoh, 1997; Durst and Loomis, 1984; Hu and Brown, 1994, Loomis and Durst, 1992; Matoh et al., 1992).

Dannel et al. (1995) prove that irrespective of B supply 1-1000 µmol L^{-1}, B concentrations in the apoplasmic liquid was generally 10 to 4 times lower than respective cell sap, demonstrating also that the presence of B inside lead cells over a large range B supplies.

Pfeffer et al. (2001) analyzed the subcellular comparmentation of B in sunflower roots, where 36% is in free space, 39% in the cell wall, 20% in the cytosol and 6% in the vacuole. Another study (Martini and Thellier, 1993) reported leaves in clover plants with total of 96% B located in the cell walls and 4% in the cytosol. It can be inferred that boron is not allocated exclusively to one single cell compartment. In fact, boron is located in all subcellular compartments with different levels. Given the chemical properties of B and the high membrane permeability for B, this element is likely to be present in all major cell compartments. The variety of results indicated in several papers show that subcellular compartmentalization of B is affected by numerous factors such as plant organ, plant species and genotypes, B supply and B status of the plant (Dannel et al., 2002).

Table 3.1 Tissue, cell and subcellular micro-localization of B in foliage of vascular plants.

Species	Exposure/duration	Tissue	Cell compartment					Reference
			Cell wall	Cytosol	Protoplast	Vacuole	Free space	
Helianthus annuus *	0.1 to 1600 µM/L	Leaf/root	+++					Dannel et al. 1998
Nicotiana tobacum L. *	1 mg/L; 1 week	Cultured cells	+++					Kobayashi et al. 1997
Nicotiana tobacum L. *	100 µM/L; 3d	Cultured cells	+++					Hu and Brown 1994
Lemna minor ^	160 µM/L; 21.5 hrs	Cultured cells	++	++		++	+	Thellier et al 1979
Helianthus annuus *	100 µM/L; 9d	Roots	++	+		+	++	Pfeffer et al 2001
	1 µM/L; 9d	Roots	+++	++		+		
Trifolium ^	16 µM/L; 3 wks	Leaves	+++	+				Martini and Thellier 1993
Chara coralin "	50 µM/L; 96 hrs	Cultured cells	++	+				Stangoulis et al 2000

* Exposure to elevated B on experimentally contaminated substrate
^ Exposure to elevated B in hydroponic conditions.
" Exposure to elevated B on aseptic conditions
+ indicate the level of accumulation

Moreover, soluble B was found to increase substantially in the intracellular compartments of leaves at high B supply. Wimmer et al. (2002) concluded that B toxicity in wheat leaves is likely to be caused by an increase in intracellular soluble B.

Populus species have been suggested as potential suited for revegetation of B in contaminate sites because of their high tolerance to extreme soil metal contamination (Bañuelos et al. 1999, 2010; Robinson et al. 2007, 2009). Rees et al. (2011, 2012) observed that the leaf B levels in poplar trees increased up to 1,350 mg kg^{-1} when increasing soil B concentrations to 13–14 mg kg^{-1}, irrespective of the B distribution. As consequence, the average shoot B concentration was found around 500 mg kg^{-1} were in the range 800–1660 mg kg^{-1}.

Boron comparmentation

Molecular mechanisms explaining boron tolerance and translocation from roots to the above-ground biomass have been identified by several authors (Takano et al. 2002; Miwa et al. 2006; Miwa et al. 2007). However, very little information has been documented related to microelement localization in leaf tissues, where most toxicity is present. Evidence may provide that no single mechanism can account for tolerance to a wide range of metals.

The study of B compartmentalization at a subcellular level has been a difficult task due mainly to limitations in the B analysis. Primarily, the detection limits are fairly high in contrast with B concentration in plant samples. Another reason is the lack of radioactive B isotopes that can facilitate the efflux studies to calculate subcellular compartmentalization of B in plant cells (Dannel et al., 2002). It is almost nonexistent the studies to analyze the visible toxicity symptoms and the presence of B in the plant tissues because of the low sensitivity for B and technical difficulties. Some successful analyses are energy dispersive X-ray micro-analysis (EDXMA), electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) to plot subcellular distribution of metals (Bringezu et al., 1999; Frey et al., 2000; Kupper et al., 1999; Nassiri et al., 1997; Wojcik et al., 2005; Zhao et al., 2000). However, the low sensitivity and interference with other ions (i.e. C) have not permitted the detection and visualization of B. Other techniques (AAS, SIMS) are appropriate to find metal storage location but no to localize metal allocation precisely.

Analyzing how the leaves allocate and detoxify metal contaminants imported through the veins may therefore help us to understand the tolerance mechanisms of plants to metal stress. The

concentration of 22 elements was measured in pooled aliquots of the total foliage. The metals leaf to cell compartmentalization was analyzed using X-ray microanalysis.

Chemically-fixed bulk-leaf samples were analyzed by energy-dispersive X-ray microanalysis (EDXMA) using field emission scanning electron microscopy (FESEM) to map several macro- and micro-elements at tissue and cell level.

The aims in this study were to asses, for three B tolerant and sensitive clones of hybrid Populus spp., (1) how much B was allocated on leaves and comparing with foliage data and (2) where B was allocated in the leaves tissues at a subcellular level to detect the associated structural changes and to characterize the main plant responses. Observed symptoms and foliage concentrations were compared with biomass parameters and metal accumulation in the different plant structures to evaluate the effect of B localization on plant performance. Results from chapter two would be compared in order to additionally investigate the B allocation strategies, and to designate symptoms of B toxicity and the distribution of B throughout the leaf at a subcellular level in Populus trees.

MATERIALS AND METHODS

Poplar trees were growth, irrigated, treated, harvested and transported as previously mentioned in chapter 2. Leaf materials were prepared and analyzed for foliage element concentration as described in Bañuelos and Akohoue (1994) and Bañuelos et al. (2010).

Sampling cryopreparation and fixation

Sample preparation and microanalysis were performed at the ZMB in the University of Zurich. Five vials containing around 5 samples each, with five to ten leaf disks replicates were stored in liquid nitrogen. Freeze substitution was prepared using Leica EM AFS2. Aluminum disks containing leaf strips were transferred into an Eppendorf tube. The time elapsed between sample extraction and transfer of plunge-frozen cells into substitution medium rarely exceeded 1 min. After transfer, vials were sealed and the specimens were cryo-substituted in anhydrous acetone at -80°C for 25 h. During this period, the medium gradually melted and the cells were chemically fixed and dehydrated. After substitution, vials were removed and allowed to come to room temperature. Samples were washed in anhydrous acetone to remove excess fixative and then dehydrated using critical-point drying (CPD) using critical point dryer

Bal-tec CPD030. CPD eliminates liquids from the sample and evades surface tension effects (drying artifacts) by never letting a liquid/gas interface to present. The technique requires the use of organic solvents such as ethanol and carbon dioxide as the intermediate fluid. The cell content was exposed by removing 100 µm shavings using a hand-microtome and mounted on aluminum stubs with carbon tape and sticky tabs and carbon coated (20 nm) in a MED 020 Bal-tec Modular High Vacuum Coating System.

Samples were also processed for transmission electron microscopy (TEM; for details, see Günthardt-Goerg et al. 1997 and Vollenweider et al. 2003). Twelve Parlier and twelve Diener samples fixed in TEM-grade buffered (pH 7.0) glutaraldehyde of which 9 samples were processed in view of TEM analyses.

EDXA

Specimens were analyzed using a variable pressure analytical FESEM (Zeiss Supra 50VP) equipped with secondary electron (SE), back scattered electron (RBSD) and X-ray (EDX) detectors. Specimens were observed at 400-3000x magnification at an accelerating voltage of 10 kV, a 30 mm condenser diaphragm, and 20 s spectrum collection times were selected to measure up to the heaviest elements in leaf tissues and prevent beam damage. Three segments with twelve Point & Shoot (P&S) measurements (from the lower epidermis to the upper epidermis) per segment have been made for each sample. Measurements were replicated three times per sample and averaged following systematic P&S measurements, based on the method in Eschrich et al. (1988). The three segments are averaged for each P&S and, normalized for Peak and background ratio (P/b).

Microanalytical measurements and spectrum analysis were interfaced by the GENESIS software (EDAX, Mahwah, USA) using the standardless procedure with automatic background subtraction, matrix correction and normalization of element data to 100% (ratio of the element intensity in sample vs. a preparation with pure element under same conditions). Total of 9 elements were measured (aluminum, boron, sodium, calcium, potassium, chloride, sulfur, magnesium and silicon).

Statistics

For the leaf samples, a 2-way factorial ANOVA was conducted using location and clone as independent factors. For the root samples, a 2-way factorial ANOVA was conducted for the factors clone and location and the interaction clone×location.

RESULTS

Soil metal concentration was influenced by the location conditions (Table 3.2). All the macronutrients (except for P) exhibit higher concentrations on all the depths in comparison with Parlier location (Figure 3.1). For the micronutrients, boron (B) showed higher accumulation for all the depths. Iron (Fe) levels are the same for both locations only at depth of 0-12 cm, and then levels decrease for Diener. Chlorine (Cl) was not detected in the Parlier location. This may be due to the mobility of Cl in soils. Cupper (Cu) showed same levels until the depth 36-48 cm, where the levels in Diener increase. Manganese (Mn) is high at depths between 0-12 cm for Diener location and then values drop. Nickel (Ni) showed the same pattern as Mn. Zinc levels are low for all the depths in Diener location in comparison with Parlier.

Table 3.2 Total macro-micro nutrients and trace elements concentrations at different soil depths

Elements		Location														
		Diener												Parlier †		
		0-12			12-24			24-36			36-48			0-12		
		Mean		SE	Mean		SE	Mean		SE	Mean		SE	Mean		SE
Cl	mg/L	1057.03	±	199.9	457.59	±	118.68	355.48	±	82.2	323.69	±	43.21	ND	±	0
Ag	ug/kg	ND	±	0	ND	±	0	ND	±	0	ND	±	0	0.24	±	0.05
Al	ug/kg	25.59	±	7.05	9.85	±	1.01	19.53	±	6.71	18.54	±	7.86	1.78	±	0.3
As	ug/kg	6.17	±	0.93	4.04	±	0.62	2.87	±	0.41	2.73	±	0.24	28.14	±	1.46
B	mg/kg	5.09	±	0.07	2.68	±	0.73	1.84	±	0.4	1.91	±	0.22	0.06	±	0.01
Ca	mg/kg	569.87	±	6.99	307.24	±	121.94	186.32	±	57.92	207.46	±	42.64	21.58	±	1.46
Cd	ug/kg	ND	±	0	ND	±	0	ND	±	0	ND	±	0	0.04	±	0.01
Co	ug/kg	2.98	±	1.27	0.18	±	0.1	0.06	±	0.04	0.07	±	0.04	1.84	±	0.41
Cr	ug/kg	0.05	±	0.03	0	±	0	-0.01	±	0.01	0	±	0	2.72	±	0.39
Cu	ug/kg	21.32	±	1.45	10.57	±	2.82	8.98	±	1.23	47.56	±	25.4	27.24	±	1.52
Fe	mg/kg	1.6	±	0.15	0.89	±	0.36	0.54	±	0.17	0.61	±	0.14	1.44	±	0.25
K	mg/kg	18.71	±	1.55	12.2	±	3.21	7.78	±	2.65	6.72	±	1.36	5.68	±	0.48
Mg	mg/kg	76.39	±	3.53	42.35	±	18.53	28.67	±	10.57	32.04	±	7.97	6	±	0.77
Mn	ug/kg	89.78	±	41.2	10.49	±	5.47	4.98	±	2.2	3.54	±	0.96	33.4	±	7.08
Mo	ug/kg	66.03	±	0.29	65.75	±	5.69	78.12	±	0.28	69.32	±	1.77	5.46	±	0.62
Na	mg/kg	1193.13	±	178.25	640.52	±	157.47	547.37	±	100.73	544.16	±	46.41	45.3	±	4.59
Ni	ug/kg	15.62	±	2.24	8.9	±	1.23	6.57	±	1.45	7.16	±	1.27	7.46	±	0.76
P	mg/kg	0.58	±	0.16	0.22	±	0.12	0.13	±	0.07	0.08	±	0.04	1.66	±	0.17
Pb	ug/kg	ND	±	0	ND	±	0	ND	±	0	ND	±	0	1.48	±	0.25
S	mg/kg	872.58	±	11.81	544.79	±	194.44	412.01	±	113.73	446.78	±	76.26	8.68	±	0.81
Se	ug/kg	12.51	±	1.95	6.87	±	2.74	3.7	±	1.46	2.79	±	0.67	0.74	±	0.04
Zn	ug/kg	0.42	±	0.18	0.32	±	0.11	1.8	±	0.95	0.23	±	0.08	7.24	±	0.56

SE= Standard error; ND= No detectable; N=6

† Parlier depths from 12-24, 24-36 and 36-48 contain generally the same concentrations of extractable elements as presented for 0-12

In trace and beneficial elements, aluminum (Al), molybdenum (Mo) and selenium (Se) have higher concentrations than Parlier. Arsenic (As) and chromium (Cr) levels in Diener are low for all depths. Chromium showed no detectable levels to negative values in Diener location. For cobalt (Co), the levels displayed the same as those at depth of 0-12 cm, and then it drops for Diener plot. Cadmium, silver and lead levels were below detection limits in Diener location.

Macronutrients

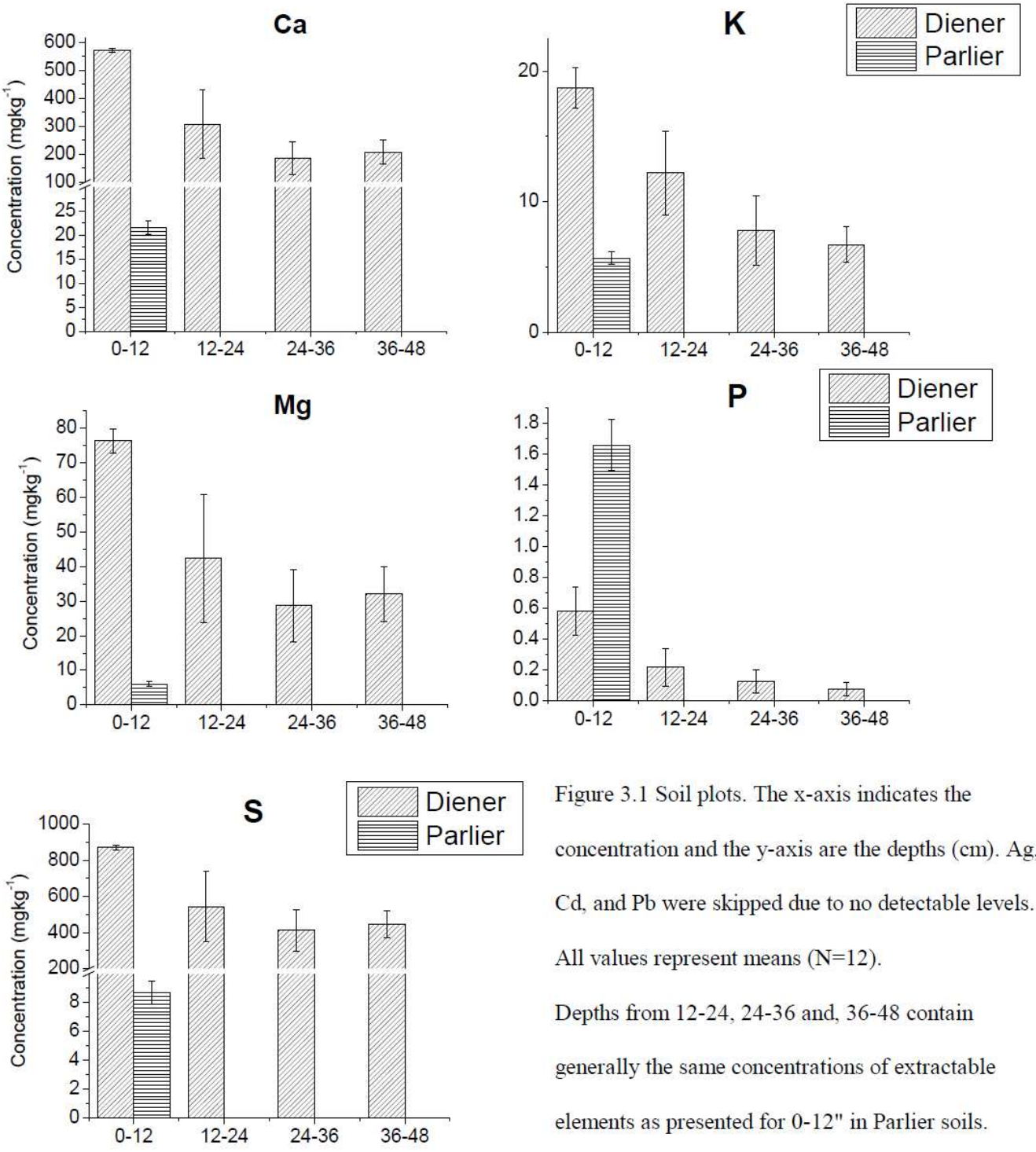
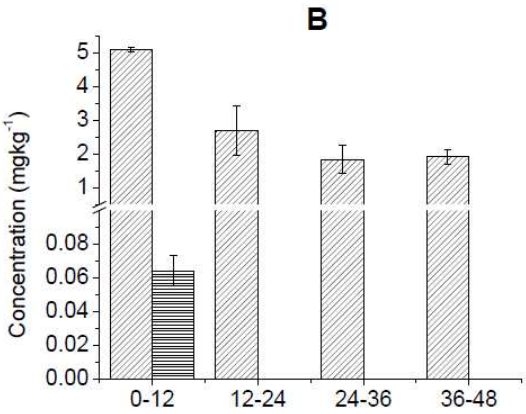
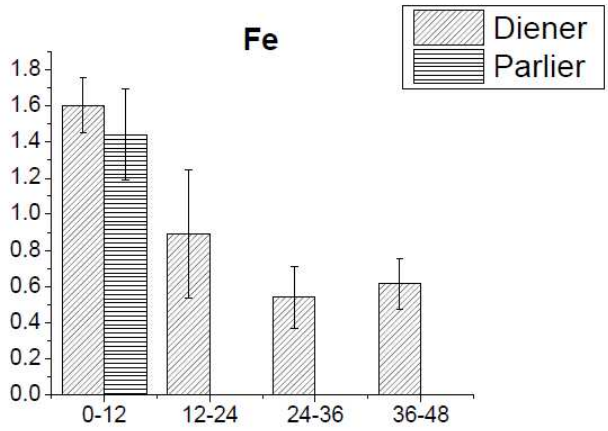


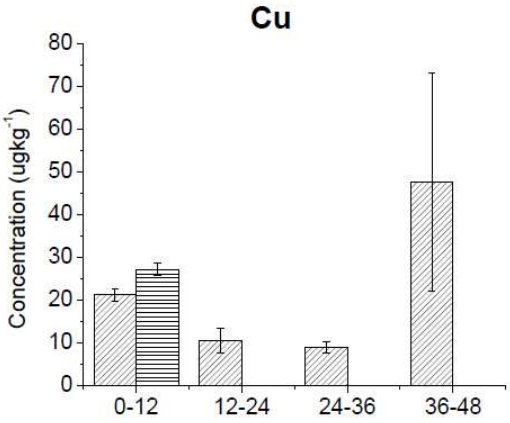
Figure 3.1 Soil plots. The x-axis indicates the concentration and the y-axis are the depths (cm). Ag, Cd, and Pb were skipped due to no detectable levels. All values represent means (N=12).

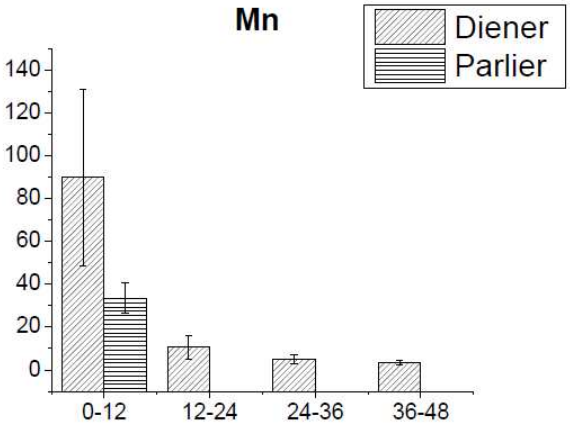
Depths from 12-24, 24-36 and, 36-48 contain generally the same concentrations of extractable elements as presented for 0-12" in Parlier soils.

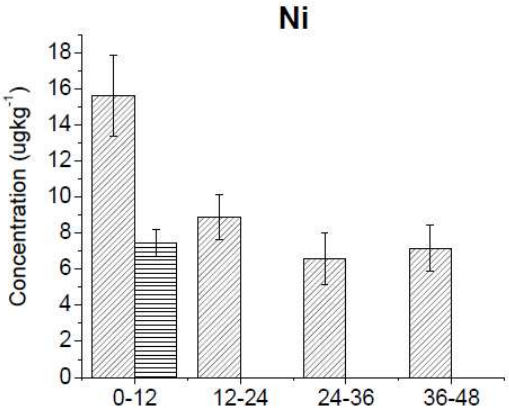
Micronutrients

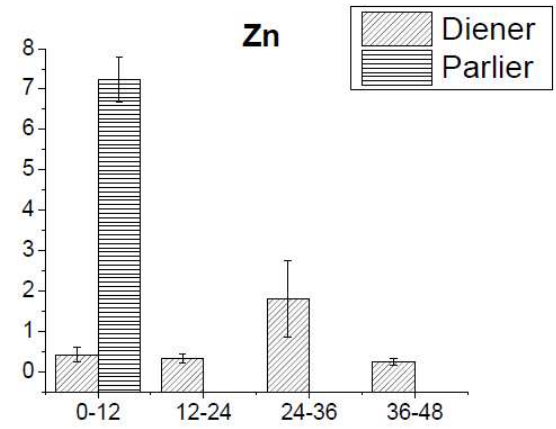


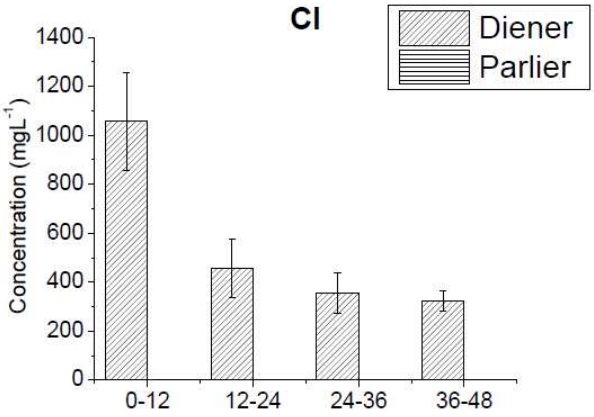




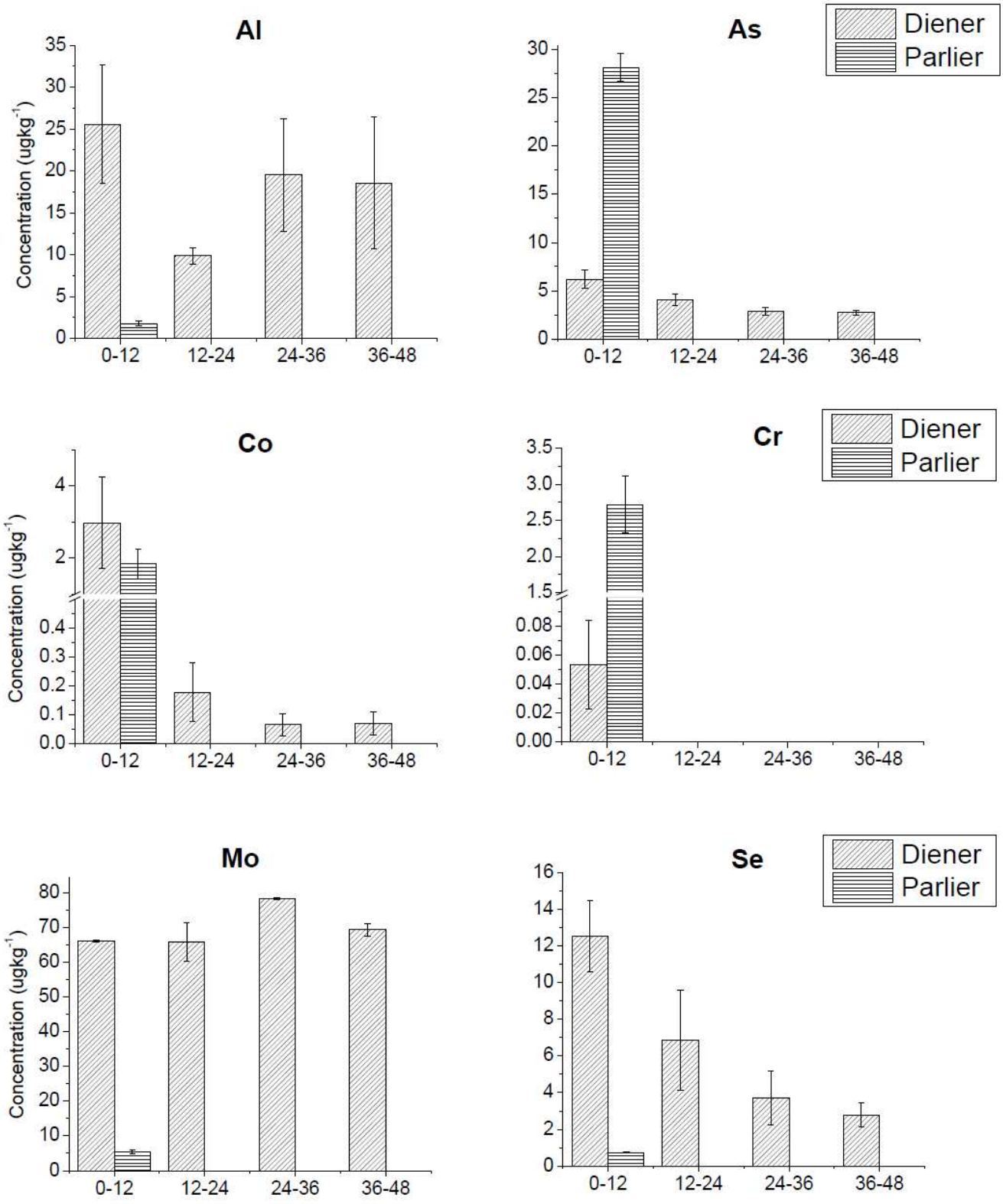








Trace elements



Tree growth on metal contaminated soil under field conditions led to significantly enhanced metal concentrations in the foliage of Populus trees (Figure 3.2, Table 3.3). The foliage concentration of aluminum, calcium (Ca), copper, molybdenum, and sulfur (S) (range Al: 0.001-0.018 mg kg^{-1}, Ca: 1500-2000 mg kg^{-1}, Cu: 4.3-7.3 mg kg^{-1}, Mo: 0.2-0.6, S: 2800-6300 mg kg^{-1}) showed the same distribution (except Mo clone 347-14 and S clone 13-366) for both locations. Concentrations for iron, potassium (K), magnesium (Mg), nickel, phosphorous (P) and lead (Pb) displayed higher concentrations in Parlier location than under field conditions. Only Fe (two-fold), Mg (two-fold) and Pb (seven-fold) showed a large difference between both locations. The rest (silver (Ag), arsenic, boron, chlorine, cadmium (Cd), cobalt, chromium, manganese, sodium (Na), selenium, zinc (Zn) exhibited higher concentrations in the Diener location. As, Cl, Cd, Co and Zn concentration on Diener's plot showed around two to three times higher concentrations in comparison with Parlier location (Cr and Mn were only a small fraction above the levels in comparison with Parlier). The greatest differences between both locations were for Na (40-fold, range 138-5688 mg kg^{-1}), Ag (33-fold, range 0.0002-0.018 mg kg^{-1}) Se (30-fold, range 0.001-1.25 mg kg^{-1}) and B (12-fold range 1580-129 mg kg^{-1}).For the analysis of variance (Table 3.4), most elements are significant different to location (except Al, Ca, Cr, Cu, Mn, Mo and S). For the factor clone, only Ag, As, Cu, Mg, Mo and PB are significant differences. This could imply that there is little difference between clones. B and Na are only significant for location. For the interaction locationxclone, only Ag and Pb are significant difference.

Table 3.3 Foliage concetrations for each clone at different locations.

Elements		Location															
		Diener									Parlier						
		345-1			347-14			13-366			345-1			347-14			13-366		
		Mean		SE	Mean		SE	Mean		SE	Mean		SE	Mean		SE	Mean		SE
Cl	[mg/kg]	10573.18	±	460.44	4107.99	±	301.36	6360.39	±	1812.04	6700.93	±	562.98	4080.96	±	317.48	2697.08	±	319.57
Ag	[mg/kg]	0.01	±	0.00	0.01	±	0.00	0.02	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Al	[mg/kg]	41.62	±	3.17	40.80	±	7.81	24.77	±	4.88	48.83	±	7.65	39.13	±	4.02	31.04	±	4.52
As	[mg/kg]	0.58	±	0.07	0.81	±	0.12	0.89	±	0.08	0.22	±	0.03	0.20	±	0.04	0.27	±	0.07
B	[mg/kg]	1232.65	±	72.04	1580.82	±	267.48	1302.32	±	154.44	195.73	±	35.70	129.59	±	8.29	198.28	±	28.99
Ca	[mg/kg]	15404.37	±	562.83	19181.59	±	1655.68	18690.95	±	3210.24	16754.58	±	1047.15	19919.22	±	657.18	17438.73	±	1255.49
Cd	[mg/kg]	0.96	±	0.09	1.03	±	0.16	1.44	±	0.31	0.31	±	0.05	0.29	±	0.04	0.57	±	0.17
Co	[mg/kg]	1.76	±	0.21	1.61	±	0.24	2.30	±	0.46	0.47	±	0.04	0.51	±	0.04	0.90	±	0.02
Cr	[mg/kg]	0.25	±	0.04	0.19	±	0.05	0.12	±	0.04	0.17	±	0.06	0.09	±	0.03	0.09	±	0.05
Cu	[mg/kg]	7.30	±	0.71	5.63	±	0.14	4.83	±	0.35	6.34	±	0.41	5.60	±	0.54	4.38	±	0.36
Fe	[mg/kg]	104.72	±	11.08	98.13	±	8.12	90.43	±	13.80	222.95	±	24.13	201.43	±	16.91	171.52	±	16.22
K	[mg/kg]	10912.35	±	1002.94	9717.49	±	945.09	5898.04	±	502.27	17509.29	±	2815.69	13251.60	±	1605.71	16284.25	±	2648.05
Mg	[mg/kg]	2990.10	±	347.92	3580.94	±	301.78	2681.13	±	292.24	4374.30	±	612.40	6199.22	±	586.29	4378.20	±	370.05
Mn	[mg/kg]	59.13	±	4.77	67.50	±	18.68	75.64	±	16.63	31.89	±	1.94	43.00	±	5.13	74.60	±	9.61
Mo	[mg/kg]	0.30	±	0.06	0.32	±	0.04	0.20	±	0.01	0.29	±	0.05	0.60	±	0.05	0.29	±	0.01
Na	[mg/kg]	5538.30	±	1036.06	4449.47	±	1295.72	5688.20	±	1317.48	159.55	±	11.87	152.22	±	21.40	138.54	±	28.92
Ni	[mg/kg]	6.63	±	1.68	5.00	±	0.84	4.83	±	1.07	9.62	±	1.13	8.18	±	0.79	7.66	±	0.86
P	[mg/kg]	1099.08	±	84.05	1270.96	±	44.01	1239.29	±	14.82	1398.35	±	134.42	1461.48	±	131.51	1739.82	±	120.01
Pb	[mg/kg]	0.11	±	0.01	0.09	±	0.01	0.08	±	0.01	0.76	±	0.07	0.67	±	0.07	0.45	±	0.03
S	[mg/kg]	2880.08	±	484.32	3910.76	±	731.51	6351.25	±	550.14	2701.91	±	126.80	3307.57	±	253.42	3969.32	±	362.48
Se	[mg/kg]	1.02	±	0.18	1.08	±	0.22	1.25	±	0.25	0.01	±	0.00	0.01	±	0.00	0.04	±	0.01
Zn	[mg/kg]	37.89	±	8.50	46.55	±	9.36	42.58	±	9.51	11.13	±	0.63	12.45	±	1.00	16.20	±	3.20

SE= Standard error; N=4

Table 3.4 The statistical significance, according to the analysis of variance, of the effects of location, clone and the interaction location and clone (- indicates P ≥ 0.05)

	Location	Clone	Location*Clone
Cl	-	-	-
Ag	**< 0.001**	**.001**	**.001**
Al	-	-	-
As	**< 0.001**	**.040**	-
B	**.001**	-	-
Ca	-	-	-
Cd	**.033**	-	-
Co	**.011**	-	-
Cr	-	-	-
Cu	-	**.002**	-
Fe	**< 0.001**	-	-
K	**.003**	-	-
Mg	**< 0.001**	**.025**	-
Mn	-	-	-
Mo	-	**.047**	-
Na	**.007**	-	-
Ni	**.042**	-	-
P	**.002**	-	-
Pb	**< 0.001**	**.001**	**.002**
S	-	-	-
Se	**.005**	-	-
Zn	**.018**	-	-

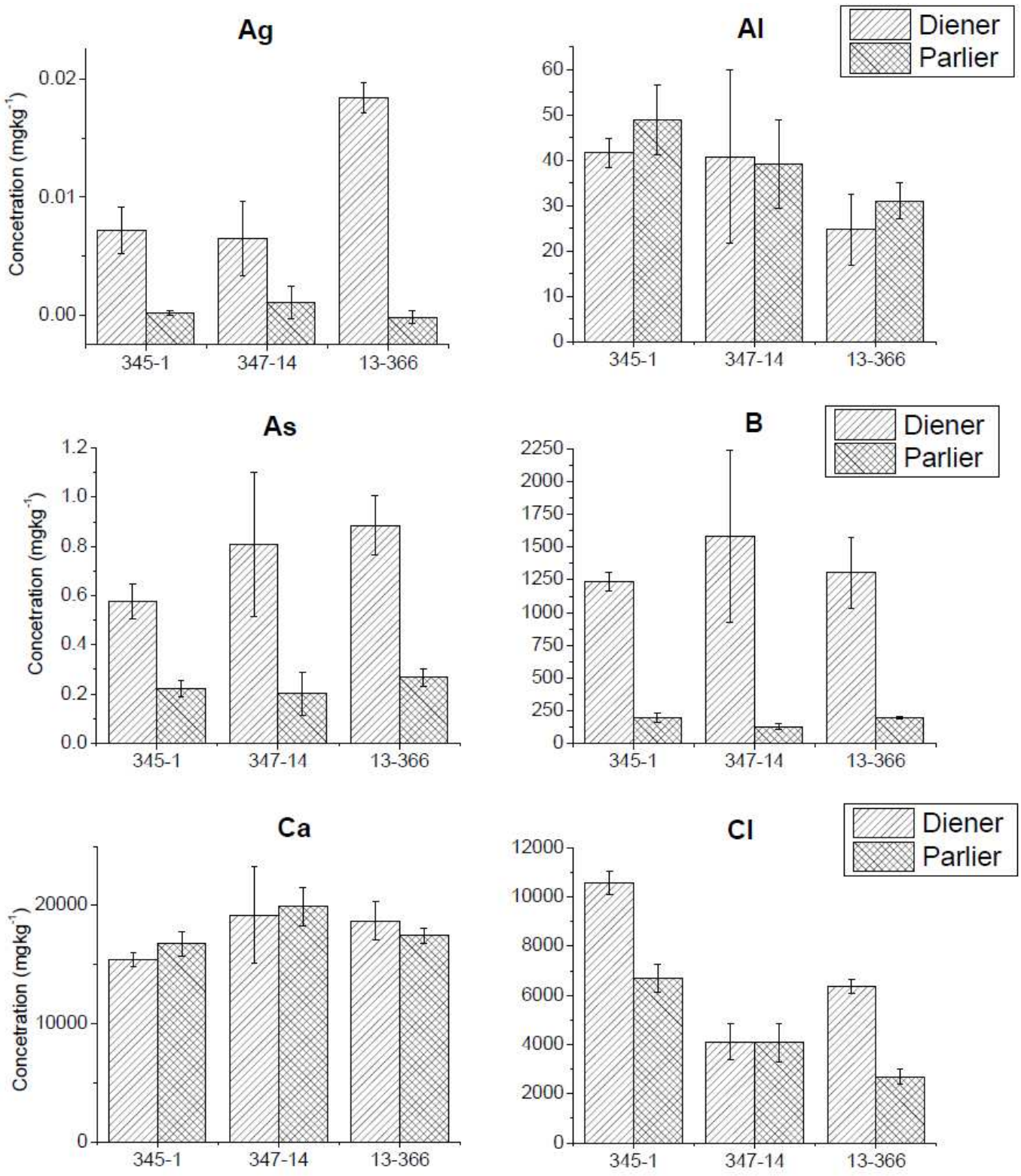
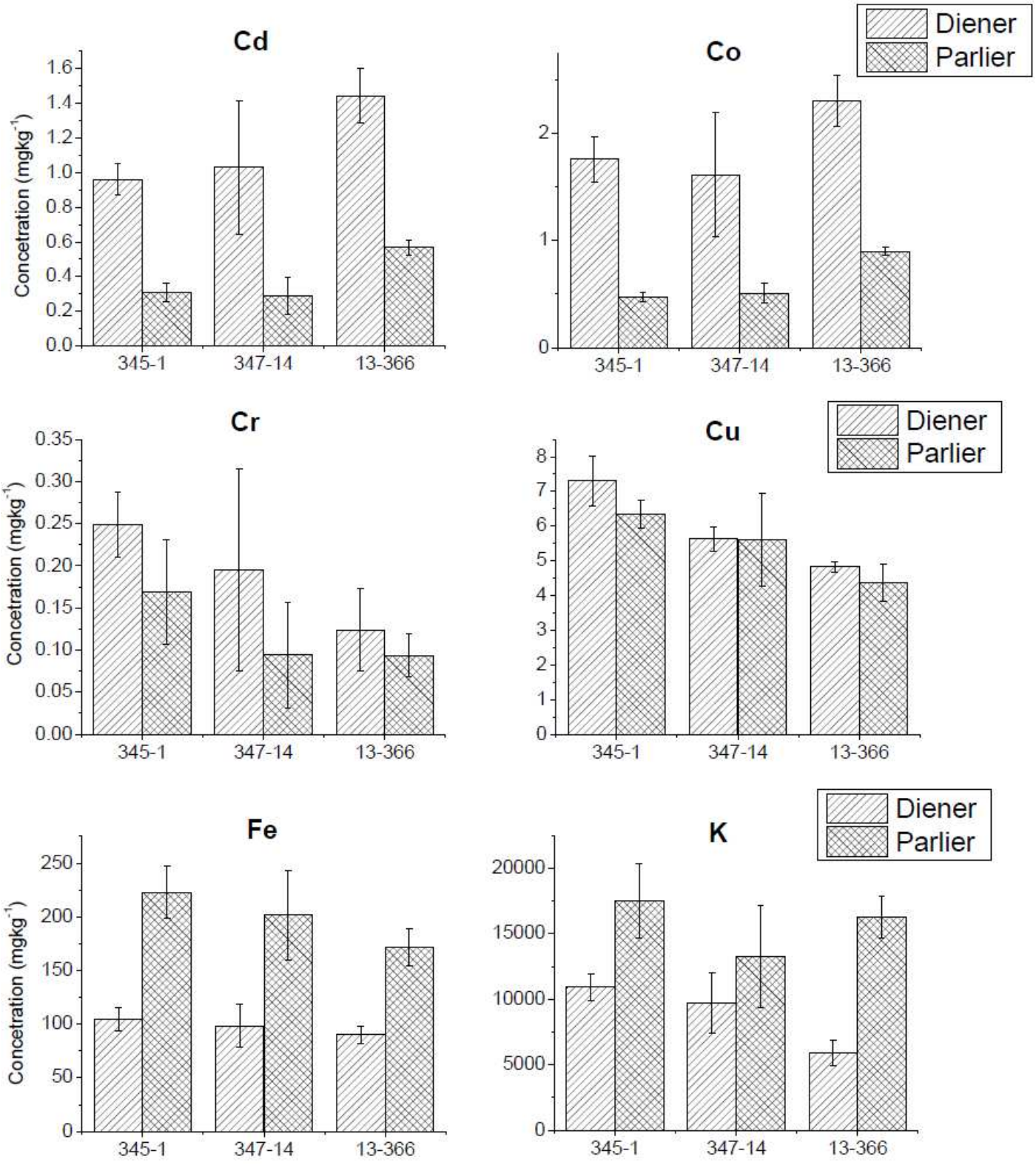
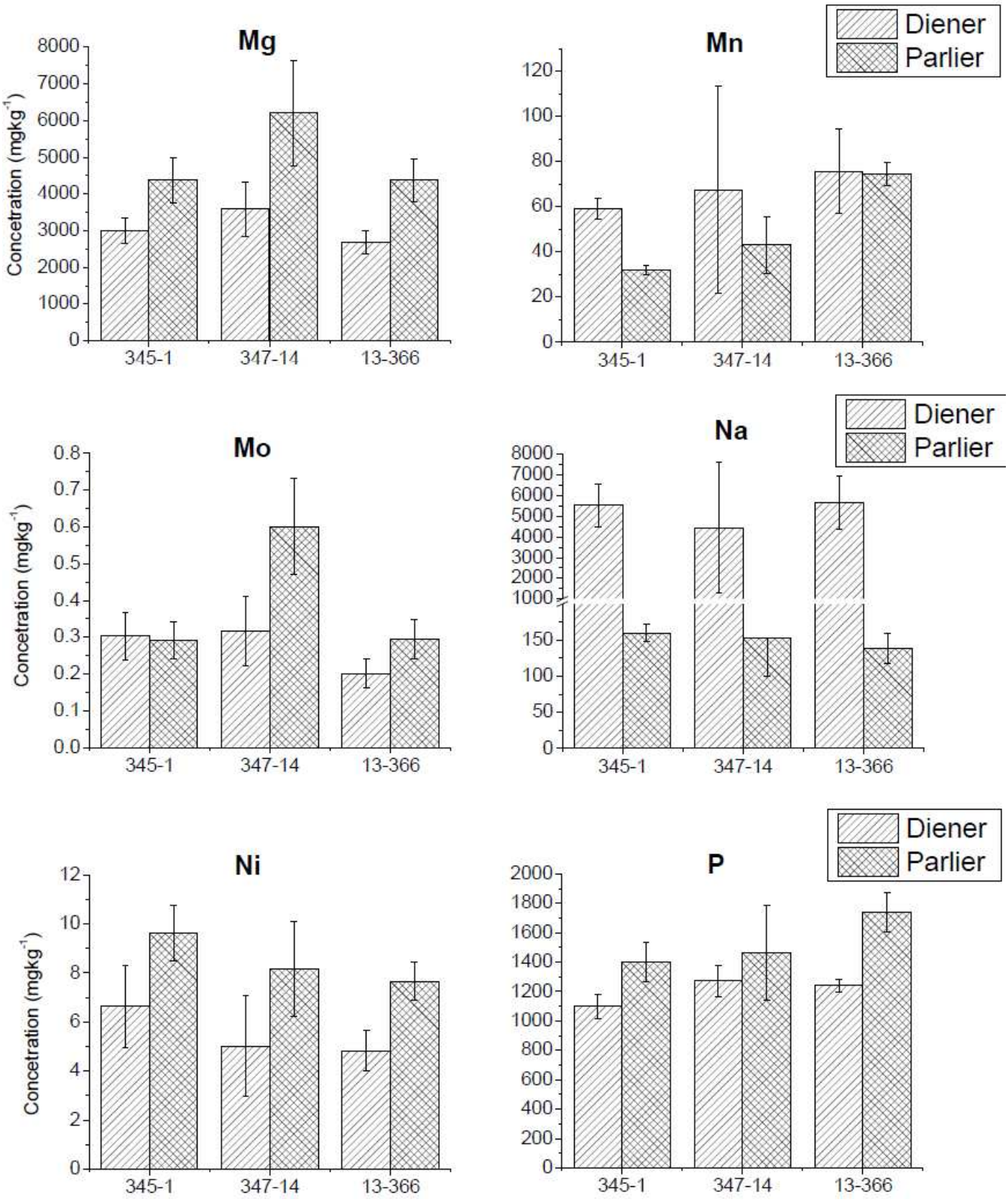
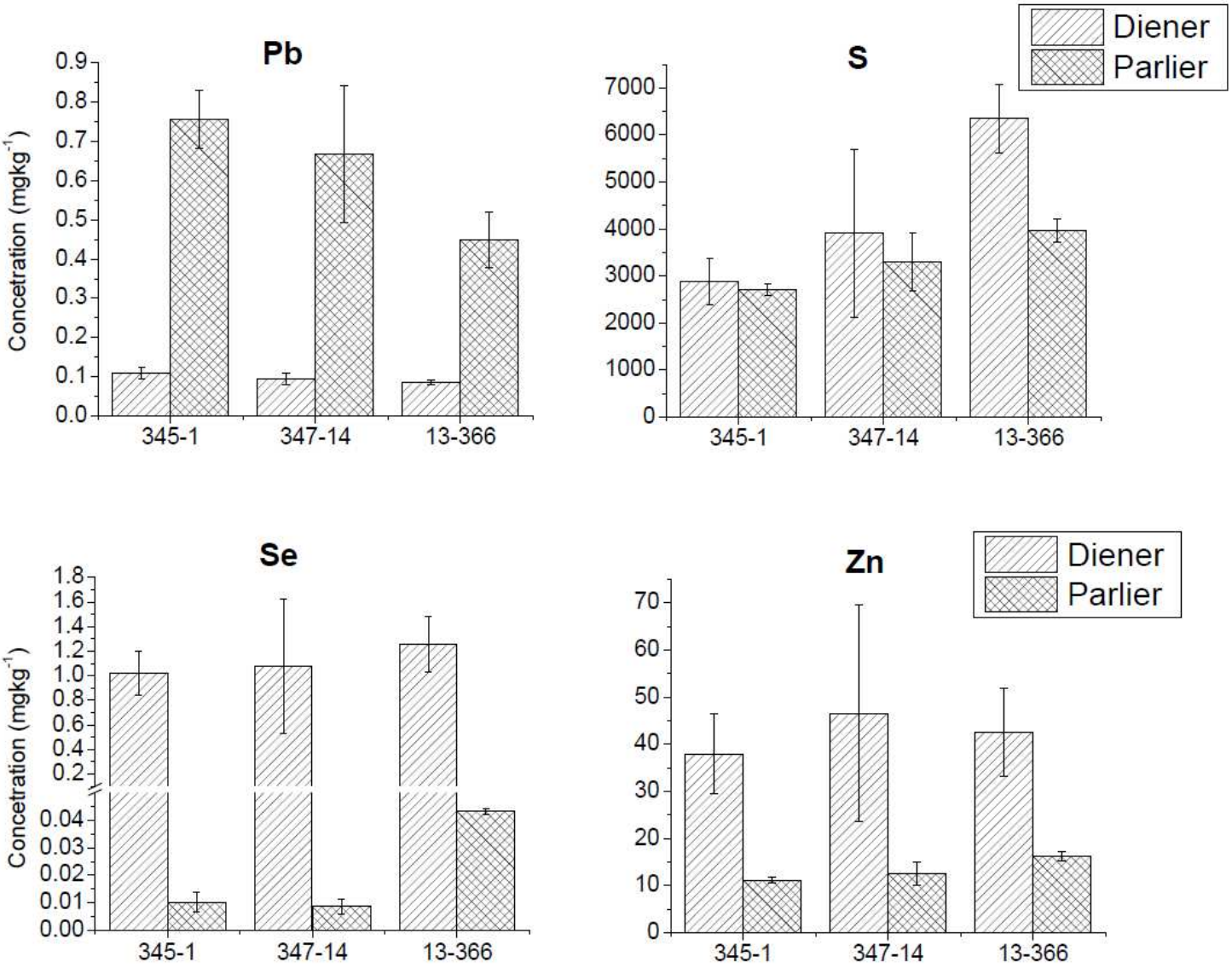


Figure 3.2 Foliage concentrations in poplar clones. All values are means (Diener, with treatment vs Parlier, control) and bars are standard error (N=12)


Cd
Co
Cr
Cu
Fe
K
Diener
Parlier
Concetration (mgkg-1)
345-1
347-14
13-366


Mg
Mn
Mo
Na
Ni
P
Diener
Parlier
Concetration (mgkg-1)
345-1
347-14
13-366


Pb
Concetration (mgkg-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
345-1
347-14
13-366
S
Diener
Parlier
0
1000
2000
3000
4000
5000
6000
7000
345-1
347-14
13-366
Se
Concetration (mgkg-1)
0.00
0.01
0.02
0.03
0.04
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
345-1
347-14
13-366
Zn
Diener
Parlier
0
10
20
30
40
50
60
70
345-1
347-14
13-366

Metal and boron allocation

Due to technical difficulties, such as the low sensitivity for boron and because of their rough surface and unstandardized thickness, critical point dried material was unfit for quantitative microanalysis. Boron mostly appears as a shoulder against the C peak (Figure 3.3A). Peak count is low and often close to background level. The results have to be interpreted as tendencies.

Element detection permitted the visualization of the element of interest along the leaf blade (Figure 3.3A and 3.3B). Carbon is a structure dependent. Boron shows correlation with carbon and it shows little detectable levels. Sodium has a larger peak (>270 counts) and is independent of all other peaks (noteworthy Al). Aluminum appears occasionally but rare. Chloride is less frequent than Na but signal may relate to salt contamination. Silicon is a frequent element, sometimes with high peaks, noteworthy on both epidermises. Calcium is occasional and apparently depending on calcium oxalate crystal occurrence.

Boron levels showed same levels as those for the Parlier location and is most consistent in the spongy parenchyma (SP) for the three clones (Figure 3.4). Na exhibits higher levels for Diener and most accumulation are found the SP. Cl showed the same levels as the control location and it is found mostly in the SP and the lower epidermis (LE). Ca also showed the same levels as those in Parlier but most of Ca is accumulated in the epidermis. Mg is also accumulated in the epidermis but it displays higher accumulation than Parlier. Al is mostly accumulated in the upper epidermis (UE) without any difference between locations. K levels are below those in Parlier and it is accumulated in the hypodermis structure. P displays a higher concentration in Parlier plot and it is found mainly in the hypodermis and palisade parenchyma. S did not show any level difference between both locations and it is most predominantly found in the SP. Silicon was found with high levels on both epidermis but without any difference for both locations. Na, Ca, Mg, Al, K, P and S showed consistent results with those in foliage concentrations.

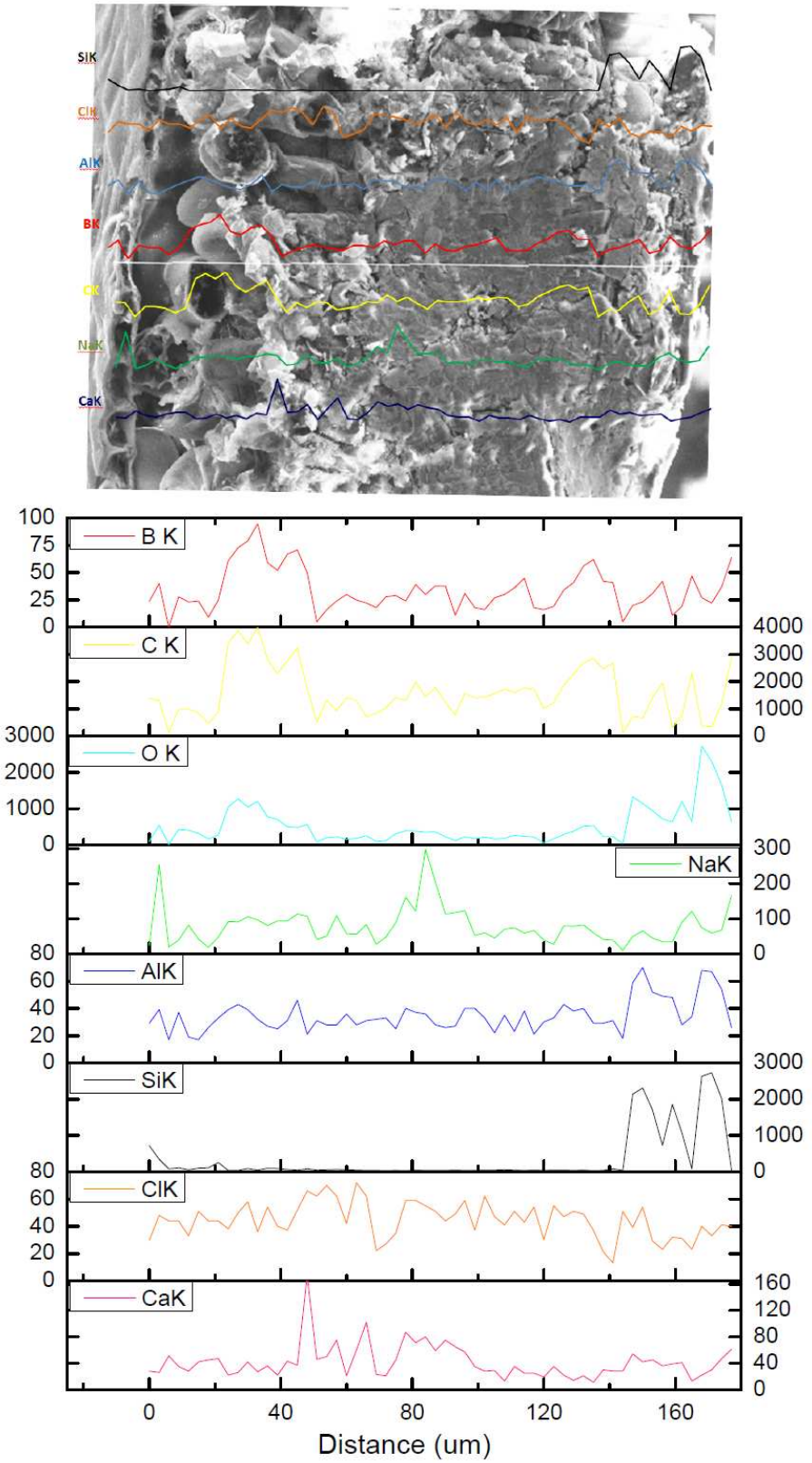


Figure 3.3A. FESEM images and Element Detection. Top, line segment analyzed and overlap element spectrum. Bottom, element spectrum for C, Na, B, Cl, Si, Ca, Al. Note: The y-axis is the number of counts it hits the element of interest. Diener sample. White line indicates the segment analyzed. From left to right, lower epidermis to upper epidermis.

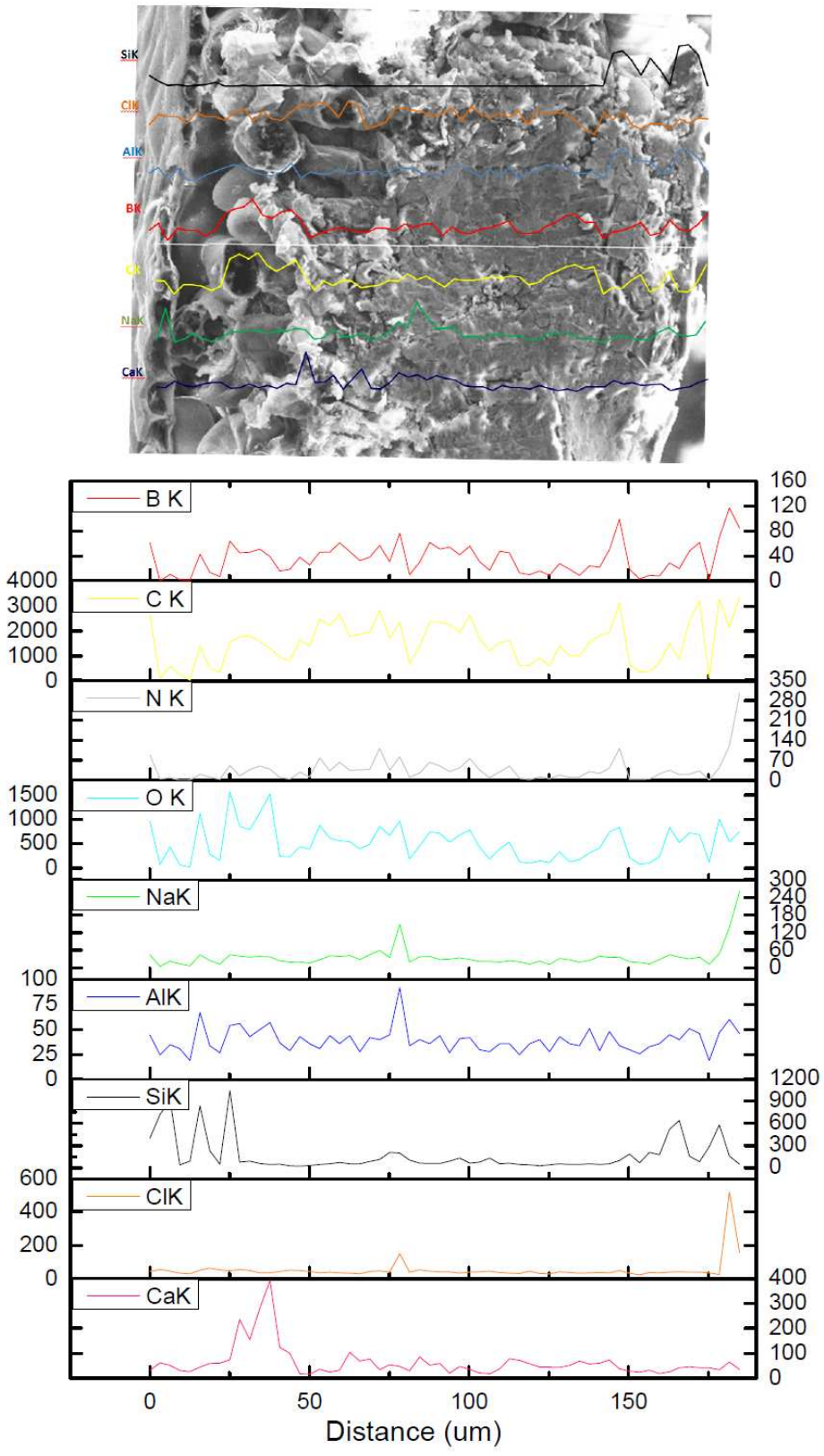


Figure 3.3B. FESEM images and Element Detection. Top, line segment analyzed and overlap element spectrum. Bottom, element spectrum for C, Na, B, Cl, Si, Ca, Al. Note: The y-axis is the number of counts it hits the element of interest. Parlier sample. White line indicates the segment analyzed. From left to right, lower epidermis to upper epidermis. Peaks beyond 175 µm are meaningless (sample surface).

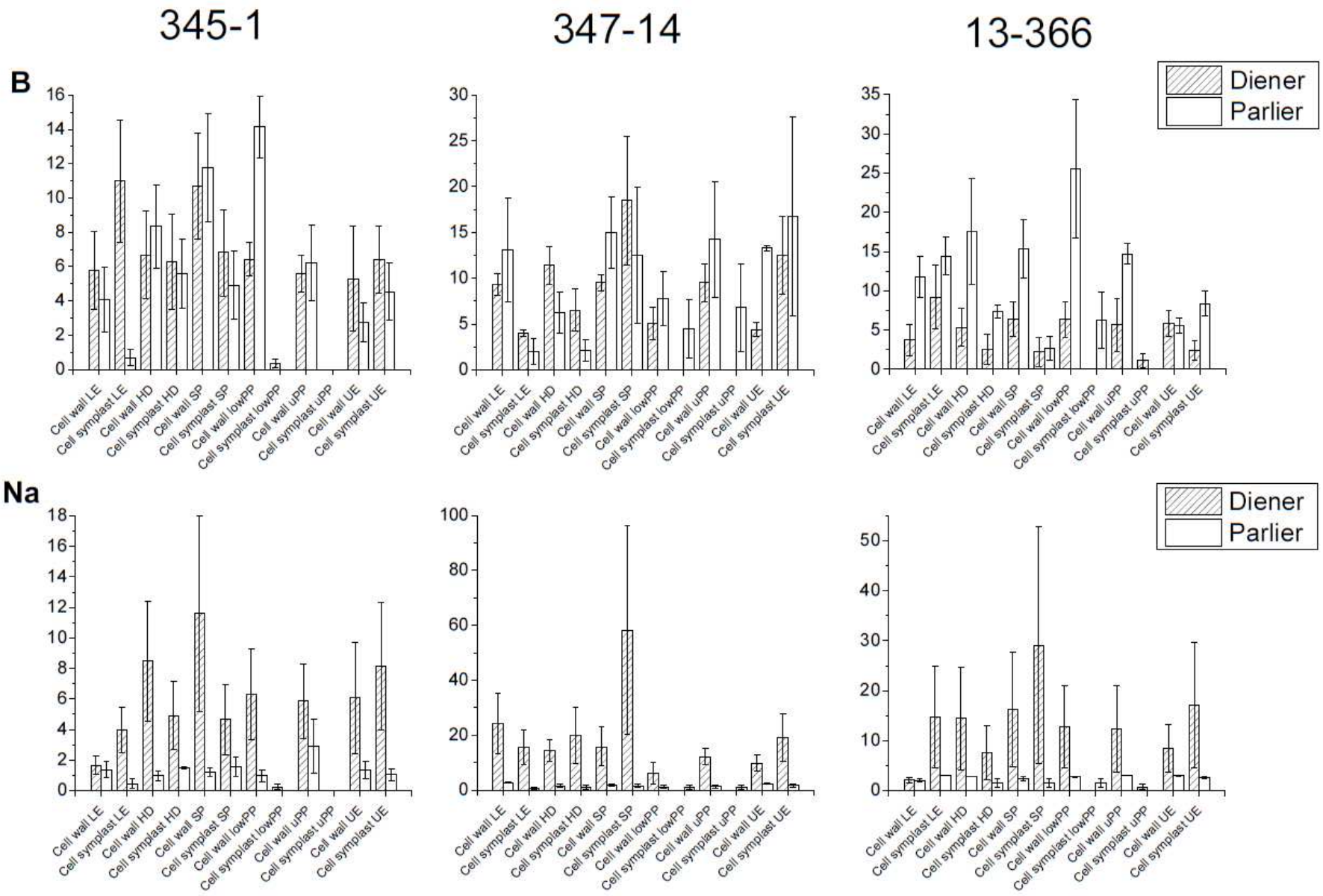
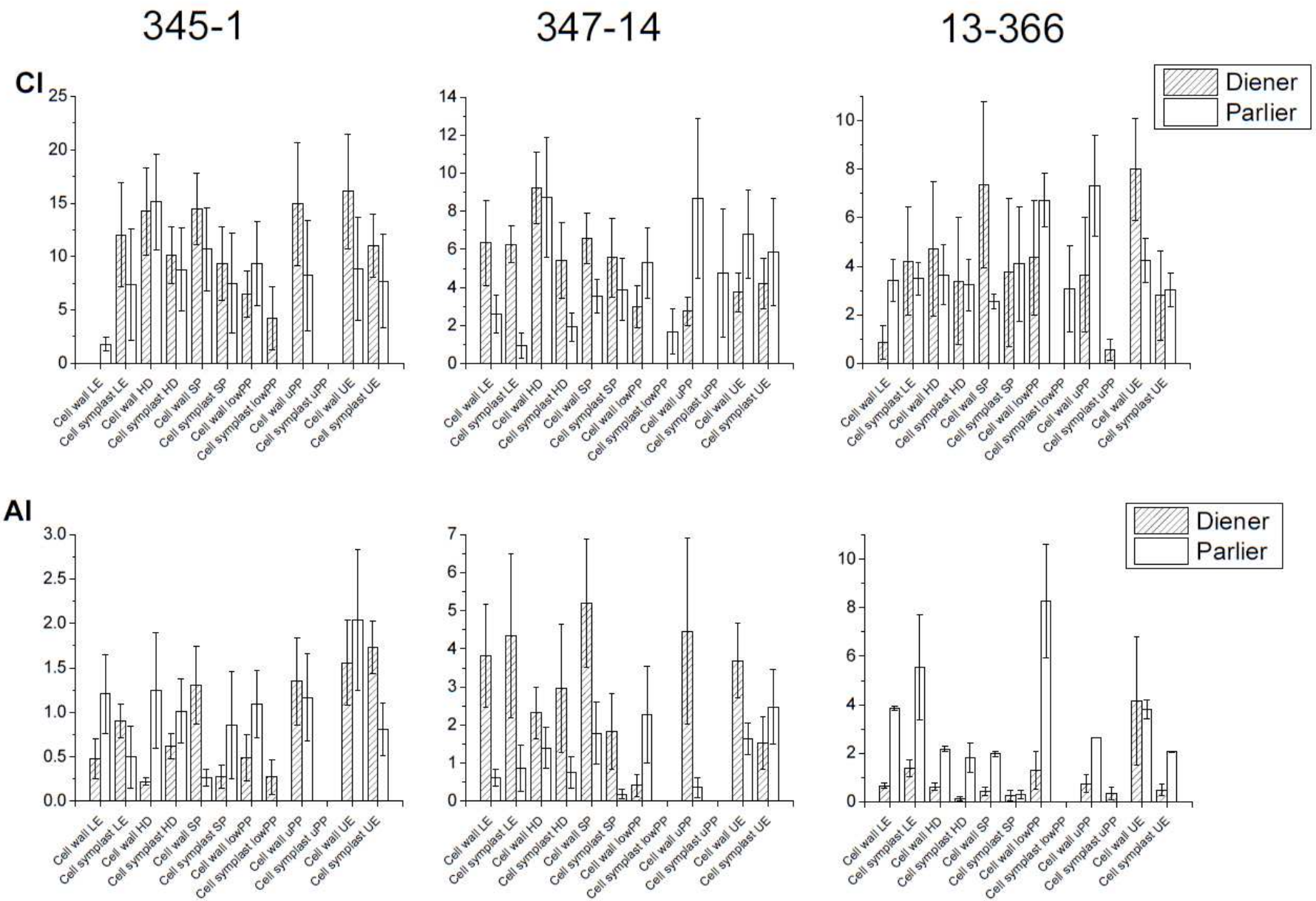
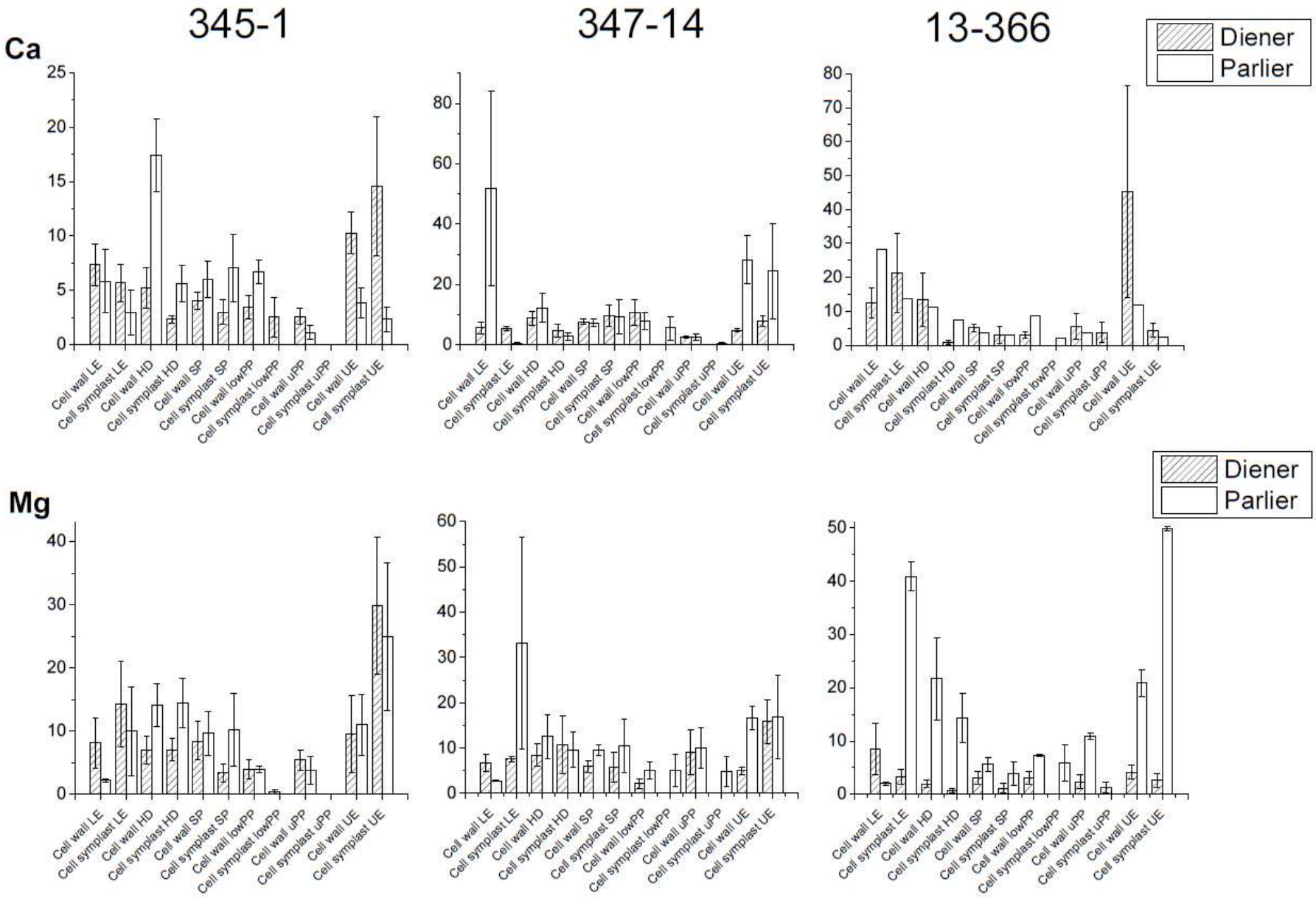
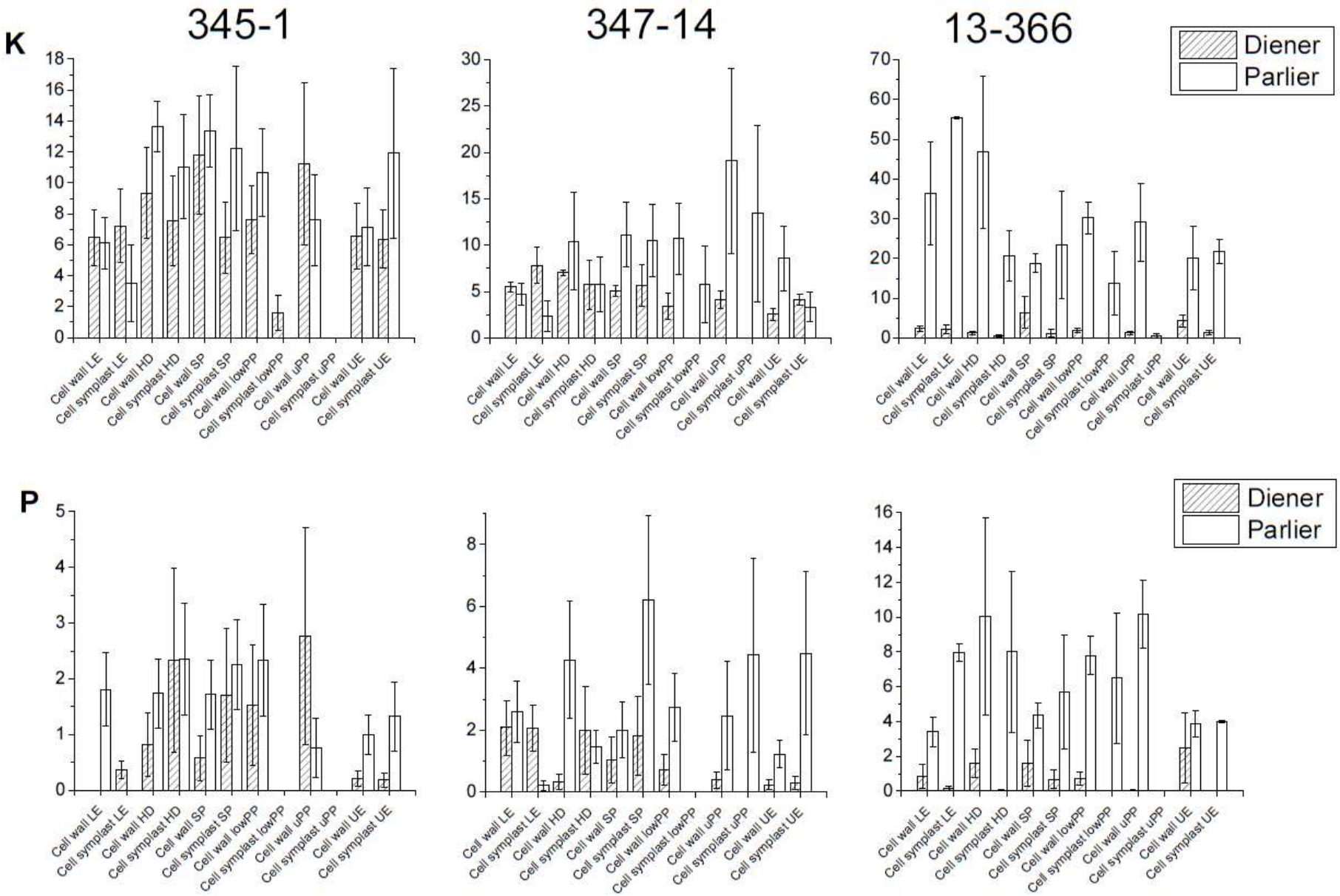
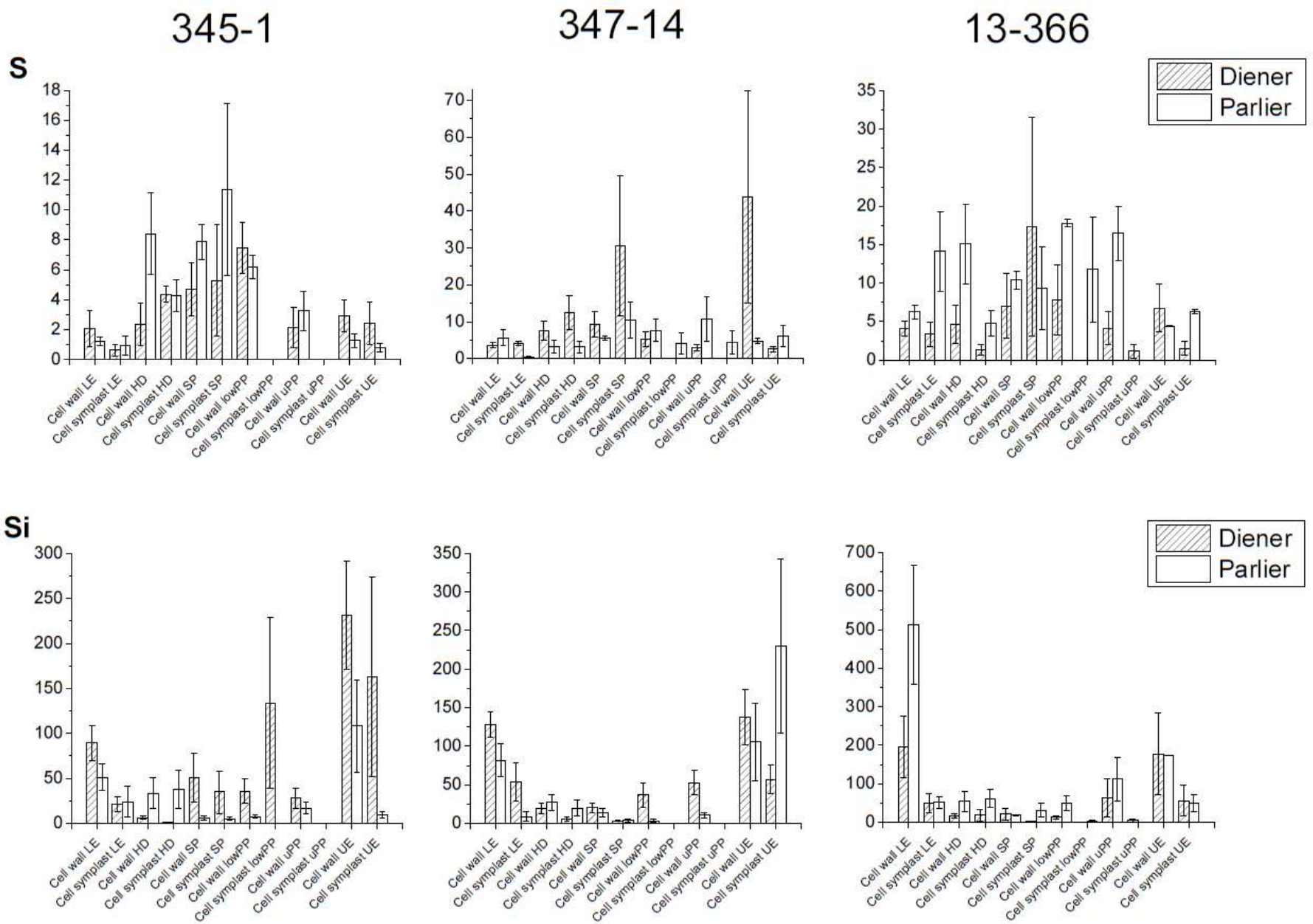


Figure 3.4 Element allocation along the leaf blade tissues. All values are means (bars represent SE). Abreviations: LE, lower epidermis; HD, hypodermis; SP, spongy parenchyma; lPP, low palisade parenchyma; uPP, upper laisade parenchyma; UE, upper epidermis


345-1
347-14
13-366
CI
AI
Diener
Parlier
Cell wall LE
Cell symplast LE
Cell wall HD
Cell symplast HD
Cell wall SP
Cell symplast SP
Cell wall lowPP
Cell symplast lowPP
Cell wall uPP
Cell symplast uPP
Cell wall UE
Cell symplast UE


345-1
347-14
13-366
Ca
Mg
Diener
Parlier
Cell wall LE
Cell symplast LE
Cell wall HD
Cell symplast HD
Cell wall SP
Cell symplast SP
Cell wall lowPP
Cell symplast lowPP
Cell wall uPP
Cell symplast uPP
Cell wall UE
Cell symplast UE


K
345-1
347-14
13-366
Diener
Parlier
P
Cell wall LE
Cell symplast LE
Cell wall HD
Cell symplast HD
Cell wall SP
Cell symplast SP
Cell wall lowPP
Cell symplast lowPP
Cell wall uPP
Cell symplast uPP
Cell wall UE
Cell symplast UE


345-1
347-14
13-366
S
Si
Diener
Parlier
Cell wall LE
Cell symplast LE
Cell wall HD
Cell symplast HD
Cell wall SP
Cell symplast SP
Cell wall lowPP
Cell symplast lowPP
Cell wall uPP
Cell symplast uPP
Cell wall UE
Cell symplast UE

Transmission Electron Microscopy

The cell content is characterized by a prominent vacuole (about 95% of cell volume) surrounded by a thin cytoplasm with a few leucoplasts, mitochondria and a nucleus showing little activity (Figure 3.5B). The vacuole appears to be filled by more or less coarsely granulated material. The cell content structure is typical for hypodermis, is similar to epidermis and contrasts with that found in mesophyll. Cells are generally necrotic or strongly degenerated as indicated by poor compartmentation and breaks in the plasmallemma, cytoplasm and tonoplast. Amorphous intercellular material and dark particles are adsorbed on the outer hypodermis thick cell wall. Hypodermis cells show a thick cell wall, except by plasmodesmata. Granular particles are adsorbed on the outer cell wall surface, mainly in the middle lamella, as they decrease in frequency towards the cell wall inner layers and they do not show up within the cells. Some cells showed evidence of autophagic processes. The adjacent epidermis cells appeared to be better preserved. Its middle lamella and outer cell walls appeared to accumulate electron-dense particles similar to those on the outer hypodermis cell wall. In contrast, the samples in the Parlier plot were similar to Diener but with more artefacts, less intercellular material accumulation, thinner cell walls and better preserved cell vitality (Figure 3.5B).

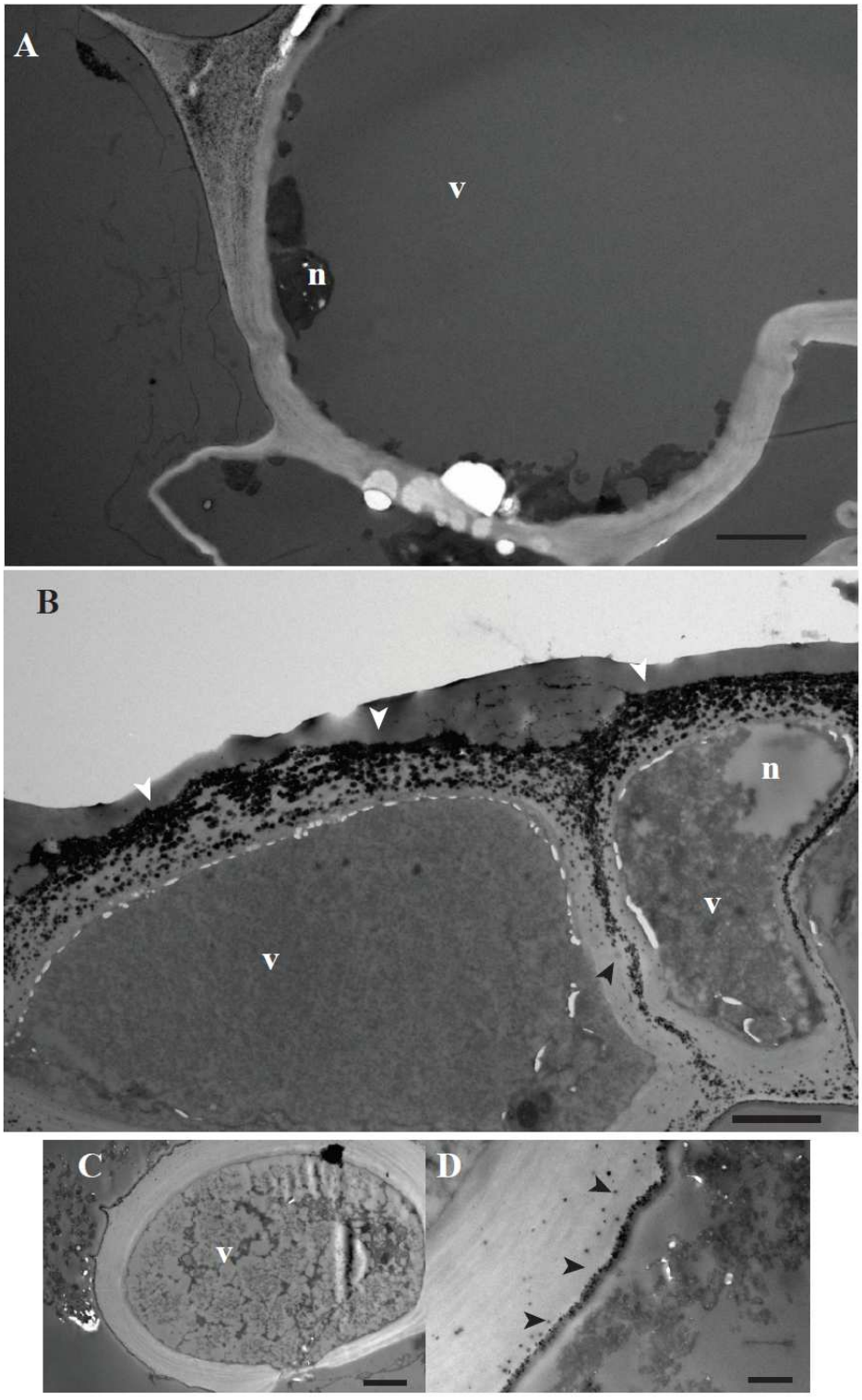


Figure 3.5 Cytological changes in the structure of assimilative cells inside the leaf mesophyll of Populus clones induced by metal contamination. Compared with parlier tissue (A), cells in the lower mesophyll (B, C, D) showed thickened cell walls. Granular particles are adsorbed on the outer cell wall surface (arrow-heads; B, E). Vacuole occupy most of the cell content (B,) surrounded with a thin cytoplasm and a nucleus showing little activity. Abbreviations: ch, chloroplasts; m, mitochondria; n, nucleus; v, vacuole. Bars: A, B, C, 2um; D, 0.5 um. Sections were examined in a TEM Philips CM 208 transmission electron microscope operated at 80 keV.

DISCUSSION

Foliage accumulation

According to Kabata-Pendias and Pendias (2001, Table 9, p. 31) metal contamination in silty and loamy soils limits on both locations remains under the acceptable range, except for Mo and Se. Molybdenum in soils for this study range 0.1-7.2 mg kg^{-1} and Se range 0.02-1.9 mg kg^{-1}. The concentrations detected for Mo reached around 80 mg kg^{-1} and 12.5 mg kg^{-1} for Se.

Also, based on Kabata-Pendias and Pendias (2001, Table 36, p. 83) and Raskin and Ensley (2000, Table 12-1, p. 194), foliage concentrations are within the normal range for most elements. Zn (Diener location) and clone 13-366 for Cu showed deficiency levels. Interestingly, Boron exhibited "excessive or toxic" levels (50-200 mg kg^{-1}) for both locations. Boron is an essential nutrient for poplar trees at leaf concentrations between 40–50 mg kg^{-1} (Mills and Jones, 1996). However, Bañuelos et al. (2010) found that no visual B toxicity symptoms were presented in all tested clones until concentrations around 300 mg kg^{-1} DM. Also, the level of B accumulation in the leaves for poplar trees is strongly correlated to water uptake (Bañuelos et al., 2010). Boron is poorly translocated inside phloem and poplars tend to accumulate high levels of elements in any conditions by the end of the vegetation season. It is hypothesized that tolerance mechanisms observed on Parlier location are based on the strong abilities for this clones to accumulate several elements. This might cause some tissue and cell injury and accelerate the senescence. Given the threshold indicated by Kabata et al. (2001) the toxic effect, if any, should be small.

In contrast to reduced tree biomass from chapter 2, the contaminated foliage shows elevated specific leaf area at the Diener's plot. This might relate to increased cell wall/defense structures or/and to higher inorganic content.

Metal allocation

The results of the microlocalization study were disappointing, as untrustworthy levels of the element of interest were detectable with FESEM. However, future developments of the technique might further improve the detectability. These complications have prevented us from obtaining reliable measurements. Careful must be taken to interpret the results obtained. The results should be considered

as potential tendencies. Boron and sodium tend to accumulate in the cell symplast and cell walls in the spongy parenchyma which in agreement with what is observed in chapter 2. Some macronutrients, such as K, P, S, and Cl also showed a tendency to accumulate in the lower mesophyll. Al, Ca, Mg and Si were found with greater accumulation mostly on the epidermis but considerable accumulation is observed along the leaf tissues. All the elements are observed within expected normal distribution across the leaf tissues. Boron as an important element in the cell wall function is observed in most cells. Excess metal concentration is managed by the spongy parenchyma cells and the hypodermis structure. The intracellular localization of B in plant cells and tentatively concluded that B is localized mainly in the apoplast and a significant amount of B does not occur in the cytoplasm (Matoh 1997; Wimmer et al., 2003). Furthermore, TEM images illustrate the tolerance mechanism (i.e. the primary allocation of contaminants to well protected and physiologically little sensitive hypodermis) identified in poplar foliage samples.

This study was carried under field conditions and multiple element contamination using three previously selected “good” performers’ hybrid poplar clones. The influence of metal contamination is not controlled and the effects presented could originate from one or more element.

In conclusion, the observations in this study identified a 2-3 cells thick layer in the lower mesophyll as specialized tolerant structure. It is not involved in essential plant activities and the main role is the handling of mineral and nutrients movement. Moreover, the cells within the hypodermis are well suited to support toxic concentrations. The minerals are accumulated and localized in the form of granular saturated complexes within the intercellular material which merely act as store houses and thus the injurious consequences of the metals cannot interference with the normal metabolism.

Balsamo and Thomson (1995) described hypodermal cells capable of function as storage reservoirs for salt and suggested its role as regulatory for salt accumulation and storage. An increased secretion of proteins, triggered by salinity, is produced in the hypodermis which may be an important site of salt sequestration away from the photosynthetic apparatus (Ball and Anderson, 1986). Furthermore, hypodermal cell were more sensitive to changes to salinity response. A similar mechanism

was observed in mangrove plants, where nitric oxide induced an increased Na^+ sequestration inside the vacuoles of the epidermis and the hypodermal cells (Chen et al., 2010).

Finally, some cautions should be considered when performing these type of studies. Careful application of washing media and washing times should avoid, or at least minimize, efflux of intracellular B into the washing solution, which can cause a misreading/miscalculations. Since the plant membrane has a high permeability to boric acid (Stangoulis et al., 2000), such as efflux is very likely to occur. More promising techniques are needed that enables direct visualization of metals in leaves, cells and subcellular organelles in situ. One of these promising techniques is Focused Ion Beam- Scanning Electron Microscope (FIB-SEM) with mass spectrometry (MS). The ion beam is used to mill material from the surface and identify the elements in a MS. The tolerance mechanisms proposed in this study should be confirmed using molecular approaches to identify potential efflux transporters in charge of handling the boron mobility within the leaf cell and leaf tissues.

Chapter 4: General Conclusions

The goals of this study were to investigate the compartmentalization, microelement localization and tolerance/detoxification mechanisms of contaminants at tree crown and leaf tissues, at a sub-cellular level to identify the strategies and cellular structures in charge of controlling the stress reactions in previously selected hybrid tolerant populus trees. The achievements of this research are summarized as follows:

A) Biomass and not tree height was, generally affected by high metal concentration. Most effects and visible symptoms were observed more apparent in leaf apexes and in younger leaves than in other leaf parts. Also, leaf branch position closer to the main stem and leaf tip and margins presented more contamination symptoms.

B) It was observed several stress reaction within the leaf tissues: Intercellular abiotic deposits are located on a water/mineral element route and are associated to local cell wall thickenings and wart-like protrusions - both are typical stress reactions. Condensed tannins were involved in metal chelating. Antioxidant functions from phenolics were involved in metal detoxification. Pectin-rich cell wall layer was a major sink for boron.

C) At the principal accumulation site of contaminants, coincidence of a naturally resistant tissue on the pathway of an overloaded nutrient flux, in the lower leaf blade tissues, there is a 2-3 cell layer-thick hypodermis. Hypodermis is a specialized tissue, with low cell physiological activity, and, being not involved in vital processes such as photosynthesis and assimilates transport, is an ideal site for B & salt detoxification.

D) Foliage concentrations detected several metal contaminations (noteworthy Na, B, Cl, Se). Only the elements of interest were found within the toxic levels.

E) Micro analytical analysis and histochemical observations confirmed that metals allocation have a tendency to accumulate in the lower mesophyll in the structure called hypodermis. Such structure has not been seen in this populus and non-xerophytes species.

Overall, these results have provided evidence of how, where and why the metal contamination is managed in poplar leaf tissues. The results have identified a clear group of cells within the lower mesophyll in charge of controlling and handling stress reactions. The intercellular structure works as store house were stress reactions are handled and as consequence no interfering with other important metabolic function within the leaf tissues. Future work should be aimed to implement to characterize molecular studies in order to identify the efflux transporters in this structure. Also, the development of new technologies and methods will facilitate the metal compartmentation in leaf tissues, especially for Boron.

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Curriculum Vitae

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