Assessment of Bur oak (*Quercus macrocarpa*) and Red Oak (*Quercus rubra*) for Salinity Tolerance and Propagation through Semi-Hardwood Cuttings

by

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ABSTRACT

The purpose of this study is to assess the ability of Bur oak (*Quercus macrocarpa* Michx.) and Red oak (*Quercus rubra* L.) to grow in saline urban soils and to investigate potential methods of vegetative propagation for Bur oak. Salt tolerance of Bur oak and Red oak was tested by exposing hydroponically-grown seedlings to 0, 25, 50, or 75 mM NaCl for three weeks. Although exposure to NaCl reduced root growth and caused leaf necrosis in both species, the deleterious effects were more pronounced in Red oak. After 3 weeks in 75 mM NaCl, the roots of Red oak seedlings ceased to grow completely, while root growth of Bur oak was only reduced by 40%. Salt-induced leaf injury was also more significant in Red oak seedlings grown in 25 mM NaCl. After exposure to different NaCl levels, nutrient uptake by root and their allocation to leaf tissue was also affected in both species. At all salt levels, concentration of essential nutrients like magnesium and calcium was higher in Bur oak leaves as compared to Red oak. The better performance of Bur oak to salinity was also attributed to the higher activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and dehydroascorbate reductase (DHAR), which lower the toxic levels of reactive oxygen species (ROS).

Structural modifications of the root system, such as the development of casparian bands and suberin lamellas closer to the root tip also contributed to the better performance of Bur oak to NaCl.

To test the potential of stem cutting for Bur oak propagation, semi-hardwood cuttings from trees of different age groups, mature trees (10-15 years old), juvenile trees (3-4 years old) and seedlings (1 year old), were collected in 2012 and 2013. The effect of 9 different hormone treatments on rooting was tested. Five hormonal treatments (0.1% IBA, 0.5% IBA, 0.05% IBA, 0.1% IBA+0.1% NAA and 0.5% IBA+0.25% NAA) were able to induce roots from cuttings

generated from 1 year old seedlings, and callus from cuttings of juvenile trees (3-4 years old). No callus or root formation was observed in cuttings collected from old trees (10-15 years old) at any hormonal concentrations. These results indicate the importance of hormonal concentrations and age of donor trees in producing successful semi-hardwood cuttings in Bur oak.

The establishment of vigorous root systems is paramount for transplanting oak plants. The effect of different commercially available trays and pruning on root architecture was also analyzed. The use of RootMakers® trays, promoting pruning throughout the entire length of root, produced dense and big root balls characterized by many lateral roots. This was in contrast to seedling grown on Ellepots trays where pruning was restricted to a small section of the root. These seedlings exhibited roots which were less dense and with fewer number of lateral roots.

While still preliminary, these results provide the basis for further studies on salinity and means to propagate Bur oak and Red oak in an effort to accelerate their introduction in urban areas.

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ABBREVIATIONS

ABA, Abscisic Acid

AP, Ascorbate

APX, Ascorbate Peroxidase

Ca^{2+}, Calcium

CAT, Catalase

Cl^-, Chloride ion

Cu, Copper

DHAR, Dehydroascorbate Reductase

Fe, Iron

GR, Glutathione Reductase

GSH, Reduced Glutathione

GSSG, Oxidized Glutathione

H_2O_2, Hydrogen Peroxide

IAA, Indole-3-acetic acid

IBA, Indole-3-Butyric Acid

K^+, Potassium ion

MDHAR, Monodehydroascorbate Reductase

Mg^{2+}, Magnesium

Mn, Manganese

Mo, Molybdenum

N, Nitrogen

Na^{+}, Sodium ion

NAA, 1-Naphthaleneacetic Acid

NaCl, Sodium Chloride

$^{1}O_{2}$, Singlet Oxygen Molecule

$O^{\cdot-}_{2}$, Superoxide Anion

OH^{-}, Hydroxyl radical

P, Phosphorus

ROS, Reactive oxygen species

SOD, Superoxide dismutase

Zn, Zinc

1. General introduction

Planting a few elite species for reforestation or introduction of trees in urban areas have often resulted in a reduction in tree diversity with increased risks for disease epidemics and pest outbreaks (Kouki, 1994). As an example, the outbreak of spruce bark beetles (*Scolytidae*) in Europe has led to extensive damage to spruce tree populations, was attributed to extensive monoculture practices (Grégoire, 1988). Increased threats of disease and pest epidemics caused by extensive monoculture in urban forests (Kouki, 1994) call for changes in forest management practices and a diversification of existing tree populations through the introduction of new species (Johansson, 2003; Mielikäinen and Hynynen, 2003; Jactel *et al*., 2005). Forests with high tree diversity have better resistance against diseases and insect pest outbreaks as compared to those dominated by single tree species (Finch and Collier, 2000; Mundt, 2002). Pure stands of white pine (*Pinus strobus* L.), for example, were more susceptible to damage from weevils as compared to stands with a mixed population of white pines and other hardwood trees (Belyea, 1923). Besides "individual" plant resistance, mixed tree stands with high species diversity provide an additional "associate" resistance against insect pests and diseases (Tahvanainen and Root, 1972). This "associate" resistance is the result of more diverse and abundant natural enemies of insect pests, olfactory and visual masking of the host plants, as well as physical barriers of non-host plants (Finch and Collier, 2000; Mundt, 2002; Hambäck and Beckerman, 2003).

Both urban plantations and forest areas of the Western prairies are largely dominated by ash species (*Fraxinus* spp.) (Ronald, 2006). These species are very susceptible to invasive pests like Emerald Ash Borer, which has already caused immense damage to tree plantations in USA (Poland and McCullough, 2006). Due to their winter hardiness, Bur oak (*Quercus macrocarpa*

Michx.) and Red oak (*Quercus rubra* L.) are among the best candidates to diversify tree populations in Manitoba and to reduce the risk of diseases or insect-pest epidemics (Gale and Grigal, 1987). Furthermore, studies have predicted that with a 2–3 °C rise in average temperatures, the ability of deciduous trees (including oak) to grow in cold climates will exceed that of conifers (Kuusela, 1990; Kellomäki and Kolström, 1992). Besides being well-established species in the natural forests of the prairies, the potential to adapt Bur oak, and its very close relative Red oak, to urban settings is still to be tested.

Therefore, before recommending a large-scale introduction of Bur and Red oak into urban ecosystems we need to examine their responses to environmental conditions unique to urban areas, such as salinity caused by the use of roadway de-icing salt. Additionally, because of the high variability in disease and pest resistance exhibited by seed-propagated trees, like oak, we need an efficient propagation system able to select and propagate superior genotypes with fixed and desirable traits (Dow and Ashley, 1998).

In Canada, the extensive use of salts, primarily sodium chloride (NaCl) for de-icing roads is critically increasing the salinity levels of road-side soils (Viskari and Kärenlampi, 2000). Although salinity affects the growth and development of both non-agricultural and agricultural species, the majority of the studies have examined the effect of salt stress on the latter, due to their economic values (Croser *et al*., 2001). In recent years, the increasing salinization of natural and urban forests by human practices (Renault *et al*., 2001; Ramakrishna and Viraraghavan, 2005; Zhu, 2007; Hanslin, 2011) has promoted a number of studies to analyse the effects of salt stress on tree species.

The presence of high sodium chloride (NaCl) concentrations in soils disrupts the water gradient between plant roots and soil medium (Munns and Termaat, 1986; Zhu, 2002), thus

creating osmotic stress in plants. Upon prolonged exposure to NaCl, Na^+ and Cl^- ions accumulate in plant tissue, reaching toxic levels and disrupting important physiological and biochemical processes (Castillo *et al*., 2007; Abogadallah 2010). Relatively high levels of Na^+ and Cl^- ions in roots also cause nutrient deficiencies by competing with the absorption of essential plant nutrients (Grattan and Grieve, 1999; Tester and Davenport, 2003). Furthermore, disruption in photosynthetic processes during osmotic and ionic stress increases the production of reactive oxygen species (ROS) causing oxidative stress (Hasegawa *et al*., 2000; Miller *et al*., 2010). The combination of osmotic, ionic, and oxidative stresses can ultimately result in leaf necrosis and senescence, and a delay in growth and development (Zhu 2002; Flowers, 2004; Vinocur and Altman, 2005; Carillo *et al.*, 2011).

Plant species exhibit great variations in their ability to tolerate salt stress (Flowers *et al*., 1986; Glenn *et al*., 1999) by displaying different sets of morphological and physiological adjustments to survive in saline environments (Greenway and Munns, 1980). Salt-tolerant plants (halophytes) cope with elevated concentrations of Na^+ and Cl^- either by synthesizing compatible solutes in their cytoplasm to adjust osmotic balance (Zhifang and Loescher, 2003; Chaves *et al.*, 2009) or by sequestering toxic ions in cell vacuoles (Fukuda *et al.*, 2004; Anil *et al.*, 2007). On the other hand, glycophytes (salt-sensitive plants) survive in saline environments by modifying their root structure in order to restrict the transport of Na^+ and Cl^- from the roots into the leaves (Yeo *et al.*, 1977; Khan *et al.*, 1995). These modifications generally include the formation of casparian bands and suberin lamellas in the root endodermis and exodermis in an effort to limit apoplastic movement (Enstone *et al*., 2003; Lux *et al*., 2004). Development of casparian bands and suberin lamellas close to the root tip has been associated with increased salt tolerance in many plant species (Hose *et al*., 2001; Krishnamurthy *et al*., 2009; Kronzucker and Britto, 2011).

Increased activity of antioxidant enzymes is also a well-known strategy adopted by plants to enhance salt tolerance by minimizing the toxic effects of ROS produced during salt stress (Abogadallah, 2010).

To successfully introduce Bur oak into urban areas it is paramount to develop an effective vegetative propagation system. Among the different propagation methods, propagation via cuttings is preferred due to the higher multiplication rate in a relatively short period of time. Successful regeneration of cuttings depends on a variety of factors, including environmental conditions, optimal applications of exogenous hormones, age of donor plants and time of collection (Acquaah, 2005; Sutanto, 2010).

Other common methods utilized for introducing new species into urban areas include transplanting existing trees. Bur oak is characterized by a single tap root, which hinders successful transplantation (Allen and Kuta, 1994; Row *et al*., 2012). Pruning of plant roots, in an effort to increase the root surface area and promoting lateral branching, has been reported to enhance the rate of transplantation in many tree species (Watson, 1986; Arnold and Struve, 1989a) including Red oak (Ruehle and Kormanik, 1986).

Based on the information provided above, the objectives of this thesis are to:

1) Compare the performance of Bur and Red oak plants to elevated levels of NaCl by measuring several morphological and physiological parameters including root structure and oxidative stress,
2) Conduct preliminary experiments on propagation of Bur and Red oak via cuttings by examining the effects of hormonal applications, age of the donor tree and time of collection and

3) Assess root architecture of oak plants produced by using different rooting containers with varied root pruning capabilities.

2. Literature review

2.1 Oak (*Quercus* spp.)

Oak species are commonly found in both natural forests and urban plantations of North America. Taxonomically, the genus *Quercus* (oak) belongs to the subclass of Hamamelidae, class Magnoliopsida (dicot) and order Fagales of the family Fagaceae (beech family) (Cronquist, 1988). The genus *Quercus* comprises about 500-600 species, of which only 11 are found in Canada (Farrar, 1995). Oak spp. are further divided into three main groups: the Red oak group, the White oak group, which includes Bur oak, and an intermediate group (Johnson *et al.*, 2002). Among these, only species of the red oak (*Erythrobalanus*) and white oak groups (*Lepidobalanus*) grow naturally in Canada (Farrar, 1995) (Table 1).

Overall, compared to other groups, species within the Red oak group are more sensitive to drought and other forms of stress (Sinclair *et al.*, 1987; Starkey and Oak, 1989, LeBlanc, 1998).

Table 1 Species of Red and White oak grown in Canada.

Red oaks (*Erythrobalanus*)	**White oaks (*Lepidobalanus*)**
Red oak (*Q. rubra* L.)	Bur Oak (*Q. macrocarpa* Michx.)
Black oak (*Q. velutina* Lam.)	White oak (*Q. alba* L.)
Pin oak (*Q. palustris* Muenchh.)	Swamp white oak (*Q. bicolor* Willd.)
Scarlet oak (*Q. coccinea* Muenchh.)	
Northern Pin oak (*Q. ellipsodalis* E.J. Hill)	

2.1.1 Bur oak

Bur oak (*Q. macrocarpa*) is a member of the white oak group (subgenus *Lepidobalanus*) and is believed to be the predecessor of most oak species (Reed and Davidson, 1955). It is a large, long-lived, deciduous and slow-growing tree species (Johnson, 1990). Bur oak is known to be more drought resistant, fire resistant, moderately shade tolerant and relatively flooding intolerant compared to other oak species (Farrar, 1995). It can survive in minimum average winter temperature of -30ºC for a period of one hundred and fifty days (Schaefer, 1988). Like other oak species, Bur oak is well adapted to the harsh and cold winters of the prairies and is known to be long-lived (Johnson, 1990). In Manitoba Bur oak trees of 500 (Hildahl and Benum, 1987) and 300 (Wolfe, 2001) years of age have been reported. Because of its adaptability and resistance to the harsh surrounding climate, Bur oak has been a symbol of strength and durability for hundreds of years (Lauriault, 1989).

Bur oak is a highly cross-pollinated species with trees showing high heterozygosity even if derived from a single seed source (Dow and Ashley, 1998; Craft *et al.*, 2002; Dutech *et al.*, 2005). This high heterozygosity also explains the high variation observed in the response to biotic and abiotic stress.

Besides being the most widely grown oak species in the region, Bur oak is also widespread across Canada and the eastern United States where it occupies habitats ranging from high elevation, wet riverbanks, and dry sandy slopes (Wolfe, 2001; Catton *et al.*, 2007). In Canada, it is commonly found in eastern Saskatchewan, southern Manitoba, Ontario, Quebec and New Brunswick (Lauriault, 1989). A Bur oak tree has a wide canopy spread and a deep taproot system, providing a good anchor to the soil and an effective mechanism to uptake water (Weaver and Kramer, 1932). These characteristics not only help Bur oak to avoid competition with small

prairie grasses for water but also confer drought resistance and a flexibility to adjust to various osmotic conditions (Reich and Hinckley, 1989; Abrams 1990; Bragg *et al.*, 1993).

2.1.2 Bur oak in Winnipeg

The city of Winnipeg was built around a well-established Bur oak forest (Allen and Kuta, 1994). During the plantation program in the late 1800's, some tree species like ash (*Fraxinus* spp.) and elm (*Ulmus* spp.) were transplanted within the city limits (Dafoe, 1998), while Bur oak was excluded due to its lower transplantation success. As a result, the majority of Bur oak trees in Winnipeg grow naturally (Allen and Kuta, 1994). Over the years, indigenous population of mature Bur oak trees in Winnipeg expanded quickly until 1986, when a decline in the population was observed. This was due to health and vigor problems, which included severe root necrosis (Staley, 1962; Allen and Kuta, 1994; Thomas and Hartmann, 1996). The cause of these problems was not ascribed to specific pathogens, but rather to stress conditions arising from the rapid urbanization occurring in the city, which weakened the resistance mechanisms of the trees (Ware and Howe, 1974; Sinclair *et al.*, 1987; Allen and Kuta, 1994). Trees with reduced resistance mechanisms were more prone to non-specific pests and diseases (Catton *et al.*, 2007). For example, a study conducted by Haack and Benjamin (1982) showed that compared to healthy trees, stressed oaks are more susceptible to chestnut borer (*Agrilus bilineatus*). One of the major factors contributing to the reduced health of urban trees has been the increasing soil salinity levels as a result of salt applications in winter. In Manitoba alone 40,000 ton of de-icing salt is applied on the roads every year (Davis, 2012). Kessler (1989) attributed the high rate of root necrosis observed in oak trees to excessive salt; not a new concept as previous studies linked high salinity to a decline of other urban species including maple trees (Westing, 1966; Ruark *et*

al., 1983; Dyer and Mader, 1986). While having a direct toxic effect, salt has several indirect effects ranging from alterations in nutrient uptake and metabolism to changes in water status (Kozlowski *et al.*, 1991) which all contribute to reduced growth.

Besides salt stress, the age of the tree is another important factor affecting its vigor and its resistance to biotic and abiotic agents. Allen (2000) reported that old Bur oak trees in Winnipeg are less vigorous than young trees, and are more prone to diseases and pathogen attacks (Franklin *et al.*, 1987). In addition, following stress conditions, older trees have a longer recovery time, which further reduces their chances of survival. Confirming these reports, studies of Tainter *et al.* (1990) and Sonesson (1999) found a positive correlation between disease frequency and age of trees.

Heterozygosity is another factor influencing stress tolerance in oak species. As indicated above, Bur oak is mainly propagated from seeds and because of the high cross-pollination frequency is highly heterozygous (Dow and Ashley, 1998; Craft *et al.*, 2002; Dutech *et al.*, 2005). This condition produces a tremendous amount of variability in stress responses, which is difficult to define and control. Seedlings derived from the same mother plant can exhibit contrasting responses to biotic and abiotic factors. Furthermore, resistance or tolerance to changing environmental conditions is often regulated by more than one gene, a condition referred to as "multigenic inheritance" (Dutech *et al*., 2005). Therefore, complete inheritance of a desirable phenotype to the next generation is often very difficult to obtain. As discussed in the next sections, vegetative propagation can be a solution to this problem.

2.2 Soil salinity

Soil salinity, the above-normal accumulation of one or more salts in the soil, can result in the retardation of plant growth and development (Rengasamy, 2006). Generally, a soil with electrical conductivity of its saturation extract (ECe) higher than 4 dS m^{-1} (deciSiemens per meter) is considered saline. This is roughly equivalent to 40 mM of NaCl (Chinnusamy *et al.*, 2005). However, the exact concentration above which salinity starts affecting plant growth is variable and depends on several factors including plant species, soil water content, and climatic conditions (Maas, 1986). Soil salinity has a huge impact on crop and tree production throughout the world. Throughout the world, more than 800 million hectares of land is affected by salinity (Rengasamy, 2006) and approximately 16 million hectares is present only in North America (Szabolcs, 1986; Rengasamy, 2006). The affected areas are increasing at a rate of 2 million hectares per year (Tuteja, 2007). As well as being common in arid or semi-arid areas characterized by low rainfall, salinity is often also present in humid climates due to poor water drainage and/or high water table (Eilers *et al.*, 1995). Singh and Chatrath (2001) documented that high salinity levels are present in many climates (from tropical to polar) and altitudes (from below sea level to the Rocky Mountains).

Soil salinity can develop both naturally or as a result of human-induced factors. Natural salinization is a long-term process resulting from weathering of rocks, which mainly release chlorides and sulphates (Eilers *et al.*, 1995; Munns and Tester, 2008), sea water intrusion into underground or aboveground irrigation systems (Rana and Katerji, 2000), and deposition of ocean salt by wind and rain in coastal areas (Rengasamy, 2006; Munns and Tester, 2008). Plants are usually able to adapt to natural salinization as the process is very slow. This is in contrast to human-induced salinization, a more rapid event caused mainly by faulty irrigation practices

(Zhu, 2007), deforestation, de-icing of roadways (Ramakrishna and Viraraghavan, 2005; Hanslin, 2011), and salt waste from mining operations (Renault *et al.*, 2001). Soil salinization caused by these human practices has been affecting agricultural crops and wild vegetation since 2400 B.C. (Russel *et al.*, 1965).

Faulty irrigations practices like irrigation with salt-rich water (Qadir *et al.*, 2007; Chen and Polle, 2010) and/or excess irrigation (Rengasamy, 2006) contribute to salinity in agricultural lands, by adding salts in the soil and bringing salts from deep layers to upper layers of the soil. Deforestation is also an important contributor to soil salinity. Replacement of long-rooted trees with shallow-rooted crop plants raises the water table and brings salts to the surface of the soil (Eilers *et al.*, 1995; Rengasamy, 2006).

In urban areas, the major human practice responsible for soil salinization is the application of de-icing salt. The major salt used in de-icing is NaCl along with some others like calcium, magnesium and potassium chlorides (Ramakrishna and Viraraghavan, 2005). Besides being toxic to plants at high concentrations, these ions alter the soil structure producing "sodic soils" which have impaired water and air relations (Rengasamy *et al.*, 2003).

Plant species affected by soil salinity include both agricultural crop species, as well as wild trees and shrubs. Because of their economical values, salt stress-related research has been mainly conducted on agricultural crop species (Croser *et al.*, 2001). The use of conventional breeding, genetic engineering and marker-assisted selection (MAS) has contributed to identify and increase the number of crop plants exhibiting tolerance to high salt conditions (Ruan *et al.*, 2010). Forest trees and shrubs are rarely affected by salinity under natural conditions because drainage and flushing by precipitation keep control of salt concentrations in soil (Renault *et al.*, 1998). However, their introduction in urban environments and the consequent exposure to

salinization, has raised interest in the development of new strategies to select trees and shrubs with increased salt tolerance.

2.2.1 Salinity in Canada

Canadian soils are affected by both natural and human-induced salinization. An example of natural salinization is the boreal salt pans created by salt springs near Lake Winnipegosis in Manitoba (Burchill and Kenkel, 1991). The most striking example of human-induced salinization in non-urban areas is caused by tailing waters from oil mining in Alberta (Renault *et al.*, 1998). Soil salinity in the affected areas often exceeds the electrical conductivity of 4 dS m^{-1} (Davis, 2012). Within the urban areas, major causes of salinity are de-icing practices (Environment Canada, 2001). In Canada, approximately 14 million tons of salt are used annually for de-icing roadways (Transportation Research Board, 1991; Environment Canada, 2001). McBean and Al-Nassri (1987) found that 90% of salt used in de-icing accumulates within 13 m from the road and severely affects roadside plantations (Viskari and Kärenlampi, 2000). The consequences of both natural and human-induced salinization are significant. Within the Canadian prairies, about 3.5 million ha of land under dry land cropping systems are reported to be affected by salinity (Wiebe *et al.*, 2007) and within Manitoba soil salinization has affected approximately 243,000 ha of farmland (Government of Alberta, 2013).

2.3 Salt stress

The mechanisms through which salt stress affects plant growth and development are very complicated as high salt concentrations also trigger additional (or secondary) stress responses (Munns, 2002; Zhu, 2007; Munns and Tester, 2008; Carillo *et al.*, 2011). Generally, salt stress is

manifested in four phases (Munns and Tester, 2008; Davis *et al.*, 2014). During the first phase, the high salt content in the soil interferes with water uptake and plants might experience water stress (osmotic stress). Once the salts are taken up they can directly affect several physiological and biochemical processes due to their toxicity level (ionic stress). The combination of both osmotic and ionic stress can trigger the production of reactive oxygen species (ROS), molecules which damage cellular components through their high reactivity. Finally, the above-average presence of salts in the soil can interfere with nutrient uptake and assimilation of other minerals required for normal growth and development; a condition which can have deleterious consequences for plant survival (Grattan and Grieve, 1999; Shannon and Grieve, 1999; Hu and Schmidhalter, 2005). While these different phases often overlap, they will be discussed separately.

2.3.1 Osmotic stress

Osmotic stress is caused by a decrease in osmotic potential resulting from a high solute concentration in the soil. A decrease in osmotic potential in the soil lowers the overall water potential, disrupting the osmotic gradient between soil and plant leaves, thus compromising the movement of water from the rhizosphere into the plant. Osmotic stress is generally considered one of the first events of soil salinity (Munns and Tester, 2008; Carillo *et al.*, 2011). Generally, an increase in soil salinity and accumulation of ions causes a rapid drop in soil osmotic potential, which lowers the cell water potential (Meloni *et al.*, 2001; Romeroaranda *et al.*, 2001).

Examples of salinity-induced water stress are many. Matsumura *et al.* (1998) reported a decrease in osmotic potential in leaves of chrysanthemum and Sea aster (*Aster tripolium*) plants with increased NaCl concentration. In *Urochondra setulosa* (a halophytic perennial grass), water

potential, osmotic potential, and stomatal conductance become reduced whereas pressure potential declined with an increase in salinity (Gulzar *et al.*, 2003). An increase in salt concentration also resulted in a reduction in evaporation rate in the halophyte *Sueada salsa* (Lu *et al.*, 2002).

Uptake of water and high turgor pressure in plant cells are essential for many biochemical processes, including cell division and elongation (Boyer, 1987; Cosgrove, 1987). Loss of turgidity followed by reduced division and elongation of both shoot and root cells were observed in rice (*Oryza sativa* L.) and *Arabidopsis thaliana* plants exposed to high salinity conditions (Yeo *et al.*, 1991; Cramer 2002). Similar results were also documented in maize (*Zea mays* L.) (Munns *et al.*, 2000b; Cramer, 2003) and barley plants (*Hordeum vulgare* L.) (Munns *et al.*, 2000a; Frick and Peters, 2002) grown in high salt concentrations. After a rapid reduction in the rate of elongation and division, cells try to recover and regain their original size by undertaking various osmotic adjustments including an enrichment of solutes in the cytoplasm (Lu and Neumann, 1998). These adjustments occur rapidly, but might not be sufficient to restore full growth (Läuchli and Grattan, 2007). Fricke *et al.* (2004) reported that barley (*H. vulgare*) plants subjected to water stress by salinity start compensating for the reduced rates of cell expansion and growth within 10-20 minutes.

Mechanisms through which osmotic stress reduces cell elongation are poorly understood, although they might involve mechanical and chemical mechanisms. Among mechanical mechanisms are yield threshold, which is minimum turgor pressure required for cell expansion, and/or hydraulic conductivity (Cramer 2003; Hu and Schmidhalter, 2004). Changes in threshold or hydraulic conductivity can be independent and species specific (Cramer and Bowman, 1993; Cramer, 2003). For example, an increase in apparent yield threshold was reported to be the main

reason for the reduced elongation rate experienced by salt-stressed maize (*Z. mays*) (Cramer and Schmidt, 1995; Cramer, 2003), while a change in hydraulic conductivity was found to be responsible for the reduced cell expansion observed in barley (*H. vulgare*) plants exposed to osmotic stress (Cramer, 2003).

Chemical mechanisms may also compromise cell elongation by affecting cell wall elasticity. The plant cell wall is a very heterogeneous structure and its composition is influenced by developmental and environmental cues. Modifications of its components, including intrusion of lignin and/or cross linkage of phenolic compounds and other polymers inhibit the wall's elasticity and consequently reduce cell elongation (Cosgrove, 1999; Neves *et al.*, 2010). Changes in cell wall composition have been observed in many plant species exposed to salt stress (Zhong and Lauchli, 1993; Wang *et al.*, 1997). Ortega *et al.* (2006) documented that higher levels of phenolic compounds due to increased activity of cell-wall bound peroxidases reduced leaf growth rate in Rhodes grass (*Chloris gayana*) grown under high salt. Indirect modification in wall architecture can also occur as a result of changes in apoplastic pH. Cell wall loosening is generally favored by acidic conditions activating expansins, hydrolytic enzymes severing wall polymers (Cosgrove, 1999). Independent reports suggest that salinity increases the apoplastic pH and suppresses expansin activity (Hu and Schmidhalter, 2004; Taleisnik *et al.*, 2009).

Another indirect effect of soil salinity on overall plant performance is a reduction in stomatal conductance, a process mediated by the plant growth regulator abscisic acid (ABA) (Munns, 2002; Flowers, 2004). ABA, rapidly synthesized by root cells sensing reduced water availability in the soil, is transported to the leaves through the xylem. Raising levels of ABA in leaf tissue induces the closing of the stomata by triggering partially unknown molecular mechanisms reducing the influx of K^+ and Cl^- ions into the guard cells (Pantin *et al.*, 2013). This

efficient perception mechanism frequently employed by plants grown under saline conditions is important for two reasons. Firstly, it ensures a reduction of transpiration, thus limiting water loss in situations of low water availability in the soil (Storey and Walker, 1999; Chaves *et al.*, 2009). Secondly, it excludes toxic ions such as Na^+ from photosynthetic tissues by halting the movement of ion-containing water in the xylem (Perera *et al.*, 1994; Véry *et al.*, 1998). Preservation of the photosynthetic machinery is vital for plant survival.

The reduction in cell division and elongation rates in plants under osmotic stress also involves the participation of other plant growth regulators, such as ethylene, which like ABA, is produced under stress conditions, and gibberellins, promoters of elongation. Gibberellin synthesis is inhibited in plant tissues exposed to osmotic stress (Achard *et al.*, 2006). Changes in overall hormone synthesis and signaling are the possible cause of the differential shoot-root growth rate observed in plants experiencing water stress. The higher reduction in shoot growth over root growth represents a sacrifice made by the plant with two important consequences. While limited shoot growth would reduce transpiration and retain water to dilute the salts within the cells, extensive root growth would ensure an "escape" mechanism from those areas of the soil where high levels of salt are present (Zhu, 2002; Munns and Tester, 2008).

2.3.2 Ionic stress

Ion toxicity is often experienced by plants growing in soils characterized by high salt concentration for an extended period of time (Munns and Tester, 2008). By following the movement of water in the roots, ions such as Na^+ and Cl^- are transported through the transpiration stream and accumulate in leaves (Munns, 2002). Although a small proportion of

Na^+ and Cl^- is redirected back to the roots through the phloem, the over-accumulation of both ions in the photosynthetic tissue has negative consequences (Flowers and Yeo, 1986).

Besides lowering the osmotic potential by drawing water into the apoplast (Munns, 2002; Tester and Davenport, 2003), Na^+ and Cl^- ions disrupt the synthesis of enzymes and structural proteins in leaves, interfere with the absorption of other essential nutrients from the soil, cause necrosis in mature leaves and lead to premature senescence (Hasegawa *et al.*, 2000; Khan, 2001; Munns, 2002). Both Na^+ and Cl^- are metabolically toxic and they cause burning and scorching of leaves when present in high concentrations (Shannon and Grieve, 1999). Symptoms of Na^+ ion toxicity start with marginal chlorosis followed by necrosis of the whole leaf, whereas Cl^- ion toxicity is initially visible as chlorosis at the tip of the leaves (Ferguson and Grattan, 2005).

Several studies indicate that the toxic effects of Na^+ are due to the ability of this ion to compete with K^+ ions for the binding sites of various enzymes which normally require K^+ for their activation. This competition can lead to complete enzymatic deactivation (Bhandal and Malik, 1988; Tester and Davenport, 2003). Affinity of Na^+ for K^+ binding sites also disrupts protein synthesis, as Na^+ replaces K^+ in specific sites required for the proper binding of tRNAs to ribosomes (Blaha *et al.*, 2000).

The extent of Na^+ and Cl^- toxicity is organ and species specific, and wide variations exist among species in the degree to which Na^+ and Cl^- can be tolerated. In general, 100mM NaCl is toxic for most enzymes (Greenway and Osmond, 1972; Munns and Tester, 2008). Ionic stress usually affects leaves more severely than other organs. This might be due to the fact that evaporation or transpiration further elevate the concentration of toxic ions in localized areas (Munns, 2002). As a result, perennial plants tend to accumulate elevated levels of ions even when grown under moderate salinity (Flowers and Yeo, 1986, Lutts *et al.*, 1996).

For most of crop species like wheat (Schachtman and Munns, 1992) and barley (Flowers and Hajibagheri, 2001) Na^+ is more toxic than Cl^-. In other species, such as narrow-leaf trefoil (Teakle *et al.*, 2010), soybean (Valencia *et al.*, 2008), citrus (Storey and Walker, 1999), plum (Hoffman *et al.*, 1989; Mead *et al.*, 1990), and grapevine (Tregeagle *et al.*, 2010), Cl^- ions are more toxic. These differences are ascribed to structural and anatomical modifications of the root which might preferentially restrict the uptake of specific ions (Munns and Tester, 2008).

2.3.3 Oxidative stress

Oxidative stress is caused by the over-accumulation of reactive oxidative species (ROS) produced during metabolic processes triggered by salinity and other types of stress. Under "normal" conditions the levels of ROS, like superoxide anions ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) are maintained low by the presence of detoxifying mechanisms which include several antioxidant enzymes (Apel and Hirt, 2004; Foyer and Noctor, 2005). This suppression might not be sufficient under stress conditions when the rate of ROS production favored by biochemical reactions occurring mainly in photosynthetic tissue, exceeds that of ROS removal (Zhu, 2001; Tuteja, 2007).

When over-accumulated, ROS alter a wide range of metabolic and physiological processes by oxidizing membrane proteins and lipids, damaging DNA and RNA molecules, and inhibiting the activity of various enzymes (Vinocur and Altman, 2005). The intensive damage to membrane lipids and proteins results in membrane dysfunction and can lead to death (Bohnert and Jensen, 1996). Severe distortion of cell membranes observed in tomato plants under salt stress have been ascribed to ROS (Khavarinejad and Mostofi, 1998). Damage to the

photosynthetic machinery is also caused by the accumulation of ROS, as they are mainly (but not exclusively) produced in the chloroplasts (Khavarinejad and Mostofi, 1998).

Antioxidants enzymes able to scavenge ROS are the major contributor to salt tolerance. They include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR), which act cooperatively to remove different forms of ROS. SOD converts $O^{\cdot-}_2$ to H_2O_2 which is then further detoxified by CAT. APX and GR produced inside the chloroplasts also contribute to the removal of H_2O_2 and $O^{\cdot-}_2$ through the well characterized ascorbate-glutathione cycle (Salin, 1991; Asada, 1994). In this cycle ascorbate (AP) and glutathione are converted from their oxidized forms to their reduced forms by the activity of several enzymes, including monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR).

Significant differences in expression and activity of these enzymes are often observed between salt-stressed plants and plants grown under normal conditions (Apel and Hirt, 2004; Foyer and Noctor, 2005). For example, exposure to salt in cotton increased the activity of SOD and GR, as well as the levels of total ascorbate (AP) and total glutathione (Gossett *et al.*,1994). Salt stress also increased the activities of SOD, CAT, APX, and GR in tomato (Rodriguez-Rosales *et al.*, 1999). Rios-Gonzalez *et al.* (2002) studied the effect of salinity (100 mM NaCl) on the antioxidant enzymes in maize (*Z. mays*) and sunflower (*Helianthus annuus*) seedlings and reported that the activities of GR, SOD, and CAT increased in plants exposed to high salt levels. In wheat plants grown under saline conditions, a sharp rise in the activities of APX, GR, MDHAR, and DHAR occurred in shoots, but not in roots (Meneguzzo and Navarilzzo, 1999). This suggests that activation of the antioxidant machinery is not only species, but also organ specific.

Exposure to different salts, or differences in the levels of the same salt, also elicit unique antioxidant responses. For example, Hernandez *et al.* (1999) reported that in pea low NaCl levels (110–130 mM) increased the activity of APX and MDHAR while the activities of GR and DHAR were increased at higher NaCl concentrations (130–160 mM).

Besides increasing the activity of antioxidant enzymes, high salinity conditions also up-regulate the expression of several genes encoding these enzymes. In both *Arabidopsis* and citrus, phospholipid hydroperoxide glutathione peroxidase (PHGPX) transcripts were induced by salt stress (Gueta-Dahan *et al.*, 1997; Sugimoto and Sakamoto, 1997), and a similar induction was also observed for Cu/Zn-SOD, GP, and APX in citrus plants exposed to high levels of NaCl (Holland *et al.*, 1993; Gueta-Dahan *et al.*,1997). It is therefore not surprising that manipulations in gene expression have been effective in enhancing tolerance to salt and oxidative stress (Lee *et al.*, 2001; Mittova *et al.*, 2003; Tuteja, 2007). For example, the over-expression of the *Chlamydomonas* GPX in tobacco plants increased tolerance to salt stress (Yoshimura *et al.*, 2004) and similar results were obtained in *Arabidopsis* plants up-regulating aldehyde dehydrogenase (AtALDH3) (Sunkar *et al.*, 2003).

2.3.4 Nutrient imbalances

Besides osmotic and ionic stress, salinity also causes nutrient deficiency in plants by altering nutrient uptake and inducing nutritional imbalances (Grattan and Grieve, 1999; Hu and Schmidhalter, 2005). These effects are due to a variety of factors ranging from a decline in essential nutrients in saline soils, the direct competition of Na^+ and Cl^- with other essential ions such as K^+ Ca^{2+}, and NO_3^- (Larcher, 1980), and alterations in nutrient partitioning within the plant (Francois, 1995).

For example, the reduced uptake of K^+ under saline conditions is often due the low availability of K^+ in the soil, as well as the competition of Na^+ and Cl^- ions for the binding site of K^+ in plasma membrane transporters (Janzen and Chang, 1987; Subbarao *et al.*, 1990). High concentrations of Na^+ and Cl^- can depress the levels of other ions in the soil, thus increasing the Na^+/Ca^{2+}, Na^+/K^+, Na^+/Mg^{2+}, Cl^-/NO_3^-, and $Cl^-/H_2PO_4^-$ ratios (Grattan and Grieve, 1994). Salinity-induced nutritional imbalance is especially detrimental for those metabolic processes regulated by enzymes using specific ions as cofactors (Grattan and Grieve, 1999; Tester and Davenport, 2003).

The effects of osmotic and salinity stress are often compounded. Cell plasmolysis, a result of osmotic stress, often alters plasmodesmata connections thus compromising the absorption and the translocation of important ions (Munns, 2002). A number of studies have reported sharp alterations in the distribution of Ca^{2+}, K^+ and Mg^{2+} levels in plants grown in high salt conditions (Khan *et al.*, 1999; Khan *et al.*, 2000; Khan, 2001). Exposure to salt reduced the content of Ca^{2+} and Mg^{2+} in leaves of guava, but not in other organs such as roots and stems (Ferreira *et al.*, 2001). This was in contrast to *Vicia faba* in which high Na^+ levels increased the intracellular Ca^{2+} content (Gadallah, 1999). Significant alterations in ion distributions within the plant often compromise growth and development. A very brief summary on the importance of several macro- and micro-nutrients is presented below.

2.3.4.1 Potassium (K^+)

Potassium is a key nutrient for plant growth as it is required for protein synthesis, enzyme activation, and maintenance of the water balance (Marschner, 1995). Water movement within the plant is regulated by K^+ which accumulates in the roots where it lowers the water potential and

facilitates water and mineral uptake from the soil (Marschner, 1995). A reduction in K^+ levels compromises these functions and negatively affects plant performance. Potassium is needed for the activation of more than fifty enzymes participating in protein synthesis and processes facilitating binding of tRNA to ribosomes (Blaha *et al.*, 2000). While the majority of studies have reported reduced levels of K^+ in plant tissues exposed to NaCl (Manchanda *et al.*, 1991; Graifenberg *et al.*, 1995; Ruiz *et al.*, 1997), a few have documented increased levels of K^+ (Meiri *et al.*, 1971; Cachorro *et al.*, 1993).

2.3.4.2 Calcium (Ca^{2+})

Calcium is a very important plant macronutrient required for the maintenance of the structural and functional integrity of cell membranes, the stabilization of the cell wall, the control of ion movements, and the activity of several enzymes (Rengel, 1992; Marschner, 1995). Calcium also plays major roles in the photosynthetic process by ensuring proper functioning of photosystem II and regulating the activity of NAD^+ kinase and phosphatase enzymes in the carbon reduction cycle (Brand and Becker, 1984). Reduced supply of Ca^{2+} disrupts the function of plasma membranes, and compromises root growth and the absorption of water and other essential nutrients (Cramer *et al.*, 1985; Cramer *et al.*, 1988).

In plants grown under saline conditions, availability of Ca^{2+} is reduced due to the competitive effect with Na^+ ions (Suarez and Grieve, 1988; Grattan and Grieve, 1999). In a study conducted on citrus, Ruiz *et al.* (1997) observed that concentrations of Ca^{2+} in leaf tissue of three rootstocks was reduced significantly with increased salinity. Na^+ ions also interfere with Ca^{2+} partitioning and transport within plants resulting in low availability of Ca^{2+} within meristematic tissues and buds. Calcium deficiency symptoms were also reported in buds of artichoke

(*Cynaras colymus* L.) grown under saline conditions, even when calcium uptake was high (Francois *et al.*, 1991; Francois, 1995). This was due to the relocation of Ca^{2+} in older leaves. In a similar experiment conducted in greenhouse conditions, Graifenberg *et al.* (1995) reported that salinity had no significant effects on calcium concentration or transport. These discrepancies in results between field and greenhouse conditions highlight the importance of environmental conditions on plant responses.

2.3.4.3 Nitrogen (N)

Nitrogen is one of most important nutrients required for plant growth. Many studies have shown a reduction in nitrogen uptake by plants grown under saline conditions (Pessarakli and Tucker, 1988; Feigin *et al.*, 1991; Pessarakli, 1991; Al-Rawahy *et al.*, 1992). Studies on cucumber (Martinez and Cerdá, 1989), eggplant (Savvas and Lenz, 1996), melon (Feigin *et al.*, 1987), and tomato (Feigin *et al.*, 1987) reported that high concentrations of Cl^- interferes with uptake of NO_3^- causing a reduced accumulation of NO_3^- in the shoot (Grattan and Grieve, 1999). The antagonistic effect of Cl^- on NO_3^- is reciprocal, as increased NO_3^- concentration in soils decreases Cl^- uptake and accumulation (Feigin *et al.*, 1987; Martinez and Cerdá, 1989). Bar *et al.* (1997) noticed improved growth and reduced leaf injury in Cl^- sensitive plants like avocado and citrus after applications of exogenous NO_3^-. These results might explain the higher NO_3^- influx rates in salt-tolerant cultivars of tomato and melon when compared to salt sensitive cultivars (Kafkafi *et al.*, 1992).

2.3.4.4 Phosphorus (P)

The relationship between tissue P concentration and salinity varies greatly with species and developmental stages (Grattan and Grieve, 1999). Some studies documented a decrease in tissue P content with increasing salinity (Sharpley *et al.*, 1992), while others show opposite effects. Contrasting results were also observed by Grattan and Grieve (1994) when comparing endogenous P concentrations in different growing media with high salt levels.

2.3.4.5 Magnesium (Mg^{2+})

Mg^{2+} is present in chlorophyll molecules and thus plays an essential role during photosynthesis (Cakmak and Kirkby, 2008). Numerous enzymes such as RNA polymerases, ATPases, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), protein kinases and phosphatases are induced by Mg^{2+} (Li *et al.,* 2001; Shaul, 2002). Besides these important functions, Mg^{2+} also regulates ionic balance across chloroplast and vacuolar membranes (Shaul, 2002). The maintenance of an adequate Mg^{2+} pool is therefore crucial for normal plant growth (Cakmak and Yazici, 2010; Cakmak, 2013).

Very little information is available on the effect of NaCl on Mg^{2+} uptake. In a study conducted on *Citrus* spp. grown under high NaCl levels, Ruiz *et al.* (1997) reported reduced Mg^{2+} concentrations in leaf tissue. Contrary to that, a survey conducted on several vegetable species showed little or no effects of NaCl or $CaCl_2$ on leaf Mg^{2+}, with the exception of sugar beet where Mg^{2+} decreased with increased salinity (Bernstein *et al.,* 1974).

2.3.4.6 Micronutrients

Plants require relatively low concentrations of micronutrients like Copper (Cu), Iron (Fe), Manganese (Mn), Molybdenum (Mo) and Zinc (Zn) as compared to macronutrients. However, their deficiency or excess can severely affect physiological and metabolic processes. The effect of salinity on availability and uptake of micronutrients is species and /or development stage specific (Grattan and Grieve, 1994). Salinity may have positive, negative or no effect on micronutrient concentrations. For example, increased salinity reduced Mn concentration in shoot tissue of barley (Cramer and Nowak, 1992), corn (Izzo *et al.*,1991) and peas (Dahiya and Singh, 1976), while increased Mn levels in tomato (Niazi and Ahmed, 1984). Different responses were also observed within the same species (Alam *et al.*, 1989; Al-Harbi, 1995).

Contrasting effects of high salt levels on the accumulation of Fe and Zn were reported. Increased salinity elevated Fe concentration in pea (Dahiya and Singh, 1976) while it reduced Fe levels in barley and corn (Hassan *et al.*, 1970). Similarly, salinity increased the amount of Zn in shoot tissue of citrus (Ruiz *et al.*, 1997), maize (Rahman *et al.*, 1993) and tomato (Niazi and Ahmed, 1984) while it reduced Zn levels in cucumber leaves (*C. sativus*) (Al-Harbi, 1995). Studies on the effects of soil salinity on the uptake of Mo and Cu are very scarce in literature and the few available show no significant correlations.

2.4 Effects of salt stress on plant growth

Salt stress in plants is a very complex mechanism operating at different levels. As indicated in the previous sections, osmotic stress and ionic stress are the two major consequences of soil salinity. Osmotic stress results in disturbances in water movement altering water and mineral uptake by the roots, while ionic stress is caused by the undesired accumulation of ions which

compromise normal growth and development directly, through their toxicity, or indirectly by influencing the absorption and/or allocation of other essential ions. As a result, salinity often disrupts many morphological, physiological and biochemical processes (Hasegawa *et al.*, 2000; Khan, 2001; Munns, 2002; Munns and Tester, 2008; Abogadallah, 2010) which in extreme circumstances can lead to death (Kozlowski and Pallardy, 1997; Viskari and Kärenlampi, 2000; Munns, 2002).

2.4.1 Morphological effects

With the exclusion of halophytes (plants adapted to grow under saline conditions), high salt levels cause an immediate inhibition of root and shoot growth (Renault, 2005) resulting in a significant reduction in fresh and dry weight of leaves, stems, and root tissue (Hernandez *et al.*, 1995; Chartzoulakis and Klapaki, 2000). When compared to plants grown under optimal conditions, plants grown under saline conditions often have fewer and smaller leaves, less branched shoots, as well as a reduced root length (Shannon and Grieve, 1999). Studies supporting these observations are many (Mohammad *et al*., 1998; Chartzoulakis and Klapaki, 2000; Meloni *et al*., 2001; Cramer 2003; Renault, 2005).

Increased salinity in tomato causes a reduction in leaf number, shoot weight, plant height, root length, and root surface area (Mohammad *et al.*, 1998). Similar results were reported in cotton where higher NaCl levels reduced root, shoot, and leaf growth, and increased the root/shoot ratio (Meloni *et al.*, 2001). It must be noted, however, the increased root/shoot ratio is not always observed. In Avocado trees, for example, root growth is more restricted by salinity stress than shoot growth (Bernstein *et al.*, 2004).

The overall reduction in shoot and root growth rates is thought to be a strategic mechanism adopted by plants to divert more resources (and energy) towards the protection and repair of tissues damaged by salt (Achard *et al.*, 2006; Flowers and Colmer, 2008). Salt stress delays plant growth by slowing down the rate of cell division and cell expansion (Bernstein and Kafkafi, 2002; Munns and Tester, 2008), and these effects are proportional to the levels of salts.

Very little is known about the precise effect of salt stress on the root system of plants. An overall reduction in lateral root density was reported in Arabidopsis plants grown under salt stress (Brussens *et al.*, 2000), while thicker root tips and accelerated vacuole formation were observed in other species subjected to high salinity (Carillo *et al.*, 2011). It must be mentioned that some of the observed morphological changes of the root system represent a strategy adopted by plants to either reduce the uptake of salts or prevent their loading into the xylem (Davis *et al.*, 2014).

Besides influencing root morphology, salt accumulation also reduces leaf growth and induces chlorosis, which in extreme circumstances can lead to necrosis (Ferguson and Grattan, 2005). Compared to other tissues and organs, leaves are more susceptible to salinity as salt tends to accumulate in mesophyll tissue following the transpiration stream (Munns, 2002).

2.4.2 Physiological effects

In glycophytes, salt stress results in metabolic dysfunctions ranging from decreased photosynthesis (Banuls and Primo-Millo, 1992; Kao *et al.*, 2001; Romeroaranda *et al.*, 2001), altered protein and nucleic acid metabolism (Bar-Nun and Poljakoff-Mayber, 1977), to decreased enzymatic activity (Greenway and Munns, 1980; Seemann and Critchley, 1985; Chaves *et al.*, 2009). Salt stress inhibits the synthesis of a wide range of proteins (Ramagopal, 1987) resulting

in very low total soluble protein contents (Alamgir and Ali, 1999; Gadallah, 1999; Muthukumarasamy *et al.*, 2000; Parida *et al.*, 2002). In Mulberry plants exposed to salinity, soluble protein content increases briefly before declining rapidly (Agastian *et al.*, 2000). Along with structural proteins, production of enzymes is also halted by high salt concentrations. Many independent studies revealed that leaf cells lose almost half of their total enzymatic activity when subjected to salinity (Shannon *et al.*,1994; Kozlowski, 1997). High concentrations of Na^+ ions are known to be toxic to a number of enzymes (Hasegawa *et al.*, 2000) directly, or indirectly by altering the uptake and allocation of other ions, such as $H_2PO_4^-$, Mg^{2+}, and NO_3^-, which are known regulators of enzymes in several metabolic pathways (Hu and Schmidhalter, 2005; Rivelli *et al.*, 2010). Among these enzymes are those involved in the Calvin cycle (ribulose-1,5-biphosphate carboxylase, ribulose-5-phosphate kinase, ribulose-5- phosphate isomerase, and NADP-glyceraldehyde - 3-phosphate) (Reddy *et al.*, 1992).

Elevated levels of Na^+ in leaves also have damaging effects on pigments and enzymes directly involved in photosynthesis and ROS homeostasis (Davenport *et al.*, 2005). These physiological effects might be the direct (or indirect) result of morphological distortions of thalakoid and grana membranes (Khavarinejad and Mostofi, 1998; Hernandez *et al.*, 1999). Overproduction of ROS within the chloroplasts, following salt exposure, severely compromises the overall photosynthetic machinery (Asada and Takahashi, 1987).

The osmotic disturbances resulting from high salinity in the soil alter water uptake and transpiration and subsequently decrease CO_2 availability (Iyengar and Reddy, 1996; Allakhverdiev *et al.*, 2000). Stomata are the main entrance for CO_2 and stomata conductance (a measure of their ability to regulate gas exchange) is significantly lowered in salt-enriched environments, a condition compromising the intake of CO_2 (Brugnoli and Bjorkman, 1992;

Chaves *et al.*, 2009). A sub-optimal photosynthetic rate leads to reduced growth and to many of the morphological abnormalities described in the previous section (James *et al.*, 2008).

Besides affecting the components of photosynthetic machinery directly, salinity also contributes to a reduction in the photosynthetic area by altering leaf morphology, limiting the size and number of leaves (Shannon and Grieve, 1999), damaging chloroplast structure (Khavarinejad and Mostofi, 1998; Hernandez *et al.*,1999), reducing chlorophyll content (Seemann and Critchley, 1985), and inducing premature senescence (Cramer & Nowak, 1992; Romeroaranda *et al.*, 2001).

2.5 Salt tolerance

Salt tolerance allows plants to cope with harmful effects of salt stress (ionic, osmotic and oxidative stress) without significant delays in growth and development (Shannon and Grieve, 1999). In order to survive in saline soils plants must acquire a wide range of morphological, physiological and biochemical adaptations (Greenway and Munns, 1980), which vary depending on the degree of salt tolerance (Flowers and Colmer, 2008). These adaptations, which include a reduction in leaf growth, adjustments in stomatal conductance and photosynthetic rate, variations in the number of chloroplasts per unit area, activation of the antioxidant machinery to minimize oxidative stress, and a regulation in ion movement, allow normal growth under saline conditions.

Plants differ widely in their ability to tolerate salt stress (Kutscha *et al.*, 1977; Braun *et al.*, 1978; Tal, 1986) and based on their "tolerance" characteristics they can be classified as halophytes or glycophytes. Halophytes are not only able to grow under high salinity conditions, but their performance is often increased by salts (Flowers *et al.*, 1977). A well characterized example is *Hordeum marinum* which is able to tolerate salt (NaCl) concentrations up to 450 mM

(Garthwaite *et al.*, 2005). The growth of glycophytes is greatly reduced by salinity and the majority of species falling in this group start showing stress symptoms at a NaCl concentration of 40 mM. At higher concentrations (100-200 mM NaCl) their growth is reduced or completely halted (Munns and Tester, 2008; Carillo *et al.*, 2011).

It must be noted, however, that extreme variations in salt tolerance are often observed among species within each group (halophytes or glycophytes) (Munns *et al.*, 1995; Storey and Walker, 1999). Examples of differences in growth performance under high salt conditions have been documented among different species of Acacia (Dunn *et al.*, 1994), Eucalyptus (Sun and Dickinson, 1993), Pinus (Townsend and Kwolek, 1987) and Sonneratia (Ball and Pidsley, 1995). Plants within the same species but of different provenance also showed high variations in salt tolerance (Allen *et al*., 1994; Morabito *et al.*, 1994; Saur *et al*., 1995; Farrell *et al.*, 1996). In cereals, bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum* ssp. *durum*) can only tolerate moderate salt stress but wheatgrass, their halophytic relative, is considered the most salt tolerant monocot (Munns and Tester, 2008). Tolerance to salinity may also vary within the same plant depending on developmental stage, degree of salinity, duration of exposure to salt, and environmental factors such as temperature and soil type (Bernstein and Kafkafi, 2002; Chinnusamy *et al.*, 2005; Munns and Tester, 2008). Complete tolerance to salt stress is only achieved by those plants able to cope with all aspects of salinity, i.e. osmotic, ion, and oxidative stress (Munns and Tester, 2008).

2.5.1 Mechanisms of salt tolerance

Three major strategies used by plants to tolerate high salt levels are osmotic adjustments, compartmentalization, and physiological and/or morphological modifications to exclude (or

restrict the entrance of) salt ions (Kozlowski, 1997; Tester and Davenport, 2003). Some halophytes have unique and extreme mechanisms to exclude excess Na^+ and Cl^- ions from the leaves through modified glands and bladders (Flowers and Colmer, 2008). Although mechanisms conferring salt tolerance may vary, it is generalized that halophytes mainly rely on osmotic adjustment (McKersie and Leshem, 1994; Gucci *et al.*, 1997), while glycophytes on avoidance strategies, which can be passive or active (Ashraf, 1994; Colmer *et al.*, 2005). Zhu (2007) suggested that avoidance strategies mainly consist of modification in root morphology, such as the formation of a passive membrane filtration system observed in mangrove plants grown under salinity conditions (Burchett *et al.*, 1984).

2.5.1.1 Osmotic adjustment and compatible solutes

Compatible solutes are low molecular weight and water-soluble compounds which do not interfere with biochemical and cellular functions even at high concentration (Bohnert and Jensen, 1996; Sakamoto and Murata, 2002). These compatible solutes are osmolytes produced to maintain osmotic balance (to lower the osmotic potential) and ensure continuous influx of water (Hasegawa *et al.*, 2000).

In some cases, compatible solutes can also function to protect plants against ion toxicity by preventing the denaturation of enzymes, stabilizing proteins and membranes, and scavenging ROS (Greenway and Munns, 1980; Hasegawa *et al.*, 2000; Zhu, 2001; Ashraf and Foolad, 2007).

High concentrations (over 40 mM on a tissue water basis) of compatible solutes, like proline (Kerepesi and Galiba, 2000; Khatkar and Kuhad, 2000; Singh *et al.*, 2000; Carillo *et al.*, 2008), glycine betaine (GB) (Muthukumarasamy *et al.*, 2000; Singh *et al.*, 2000; Wang and Nil, 2000; Jain *et al.*, 2001), sugars (Bohnert and Jensen,1996; Kerepesi and Galiba, 2000; Parida *et*

al., 2002), and polyols (Bohnert *et al.*, 1995; Parida *et al.*, 2002) are found in leaves of halophytes exposed to high salt. Elshintinawy and Elshourbagy (2001) reported that total amino acid content in wheat seedlings increased by 160% (as compared to control) after treatments with NaCl. An increase in soluble sugars as a result of starch degradation was observed in leaves of mangroves (Parida *et al.*, 2002) and tomatoes (Khavarinejad and Mostofi, 1998) exposed to NaCl. A rise in the activities of sucrose phosphate synthase in salt stressed rice (Dubey and Singh, 1999) and tomatoes (*Lycopersicon esculentum* L.) (Gao *et al.,* 1998) was responsible for converting complex carbohydrates into sucrose.

Gene manipulation has been successfully used to improve osmo-regulation. Plants ectopically expressing genes that enhance production of glycine betaine have increased tolerance to salinity (Chen and Murata, 2002; Sakamoto and Murata, 2002; Sulpice *et al.,* 2003; Ashraf and Akram 2009). Similarly, *A. thaliana* plants over-expressing the mannitol biosynthetic gene M6PR exhibited significant improvement in salt tolerance (Zhifang and Loescher, 2003).

2.5.1.2 Ion exclusions through compartmentalization

One strategy adopted by plants to cope with salinity is to sustain basic metabolic and biochemical processes by compartmentalizing toxic salt ions into cellular and/or tissue compartments such as vacuoles, older leaves, tracheids, and roots (Flowers and Yeo, 1986, Lutts *et al.*, 1996). When present in low concentrations, salts accumulate inside the cytoplasm where they contribute to the cellular osmotic adjustments (Munns and Tester, 2008). At higher concentrations however they become toxic and before they start compromising metabolic functions they are stored into the vacuoles (Shannon *et al.*, 1994; Iyengar and Reddy, 1996; Zhu, 2003). This process is concomitant with the synthesis and accumulation of compatible solutes in

the cytoplasm to maintain a low osmotic potential (Ashihara *et al.*, 1997; Zhifang and Loescher, 2003). Compartmentalization of both Na^+ and Cl^- ions in the vacuole has been observed in many species subjected to salinity stress (Fukuda *et al.*, 2004; Anil *et al.*, 2007). Extreme forms of adaptations are observed in some halophytes where the sequestration of salt ions is facilitated by the formation of specialized large vacuoles or glands (Flowers *et al.*, 1986).

Movement of Na^+ from the cytoplasm into the vacuole is mediated by Na^+/H^+ antiporters, such as the Na^+/H^+ exchanger (NHX), located in the tonoplast. These transporters are powered by the electron motive force generated by pyrophosphatase (AVP) and H^+-ATPases, which actively acidify the vacuole by pumping H^+ from the cytoplasm (Munns and Tester, 2008; Kronzucker and Britto, 2011). Over-expression of a Na^+/H^+ antiporter gene has been shown to increase salinity tolerance in rice (Chen *et al.*, 2007), Arabidopsis (Apse *et al.*, 1999), cotton (He *et al.*, 2005), tomato (Zhang and Blumwald, 2001), *Brassica napus* (Zhang *et al.*, 2001) and alfalfa (Li *et al.*, 2011). Similarly, over-expression of the vacuolar H^+ pyrophosphatase (AVP1) was sufficient to increase salt tolerance in Arabidopsis (Gaxiola *et al.*, 2001; Brini *et al.*, 2007). Differences in the activity of Na^+/H^+ antiporters occurring among species might relate to the observed variations in salt tolerance. While the majority of studies deal with compartmentalization mechanisms of Na^+, not much information is available on the exclusion of Cl^- (Munns and Tester, 2008; Teakle and Tyerman, 2010). What is clear, however, is that the movement of Na^+ and Cl^- in the vacuole is concomitant to an active accumulation of K^+ and compatible solutes in the cytoplasm in an effort to retain adequate osmotic values (Flowers *et al.*, 1977; Wyn Jones *et al.*, 1977).

2.5.1.3 Salt avoidance by root modification

Plant roots absorb ions and water from soils either through symplastic or apoplastic pathways. The symplastic pathway ensures a regulated intake of ions and water through selective channels present on the plasma membranes of the hypodermal, cortical and endodermal cells. While this movement is highly regulated by the cell membranes, which are able to exclude specific molecules, the apoplastic pathway involves a non-selective movement of water and ions within the intercellular spaces and along the cell wall outside the plasma membrane. The exact mechanisms of salt absorption from the soil are not fully understood (Kronzucker and Britto, 2011), and the contribution of symplastic and apoplastic pathways varies significantly from species to species. For example, apoplastic flow of Na^+ in roots of rice (*Oryza sativa*) plant is reported to be about ten times that of wheat (Garcia *et al.*, 1997), and even within the same plant the relative contributions of the two pathways to ion absorption varies with development (Melchior and Steudle, 1993; Frensch *et al.*, 1996). It is generally established that plants with increased salt tolerance tend to develop more selective symplastic paths, while reducing the apoplastic intake (Yadav *et al.*,1996; Yeo *et al.*,1999). This strategy would ensure continual supply of water and nutrient essential for proper growth and development while restricting undesired and toxic ions.

Almost all plants are known to develop casparian bands on the anticlinal cell walls of the root endodermis by deposition of suberin and lignin (Schreiber *et al.*,1999). Casparian bands function as a barrier to the radial movement of ions and water reaching the stele apoplastically, forcing their symplastic intake (Schreiber *et al.*,1999; Zimmermann *et al.*, 2000; Bucking *et al.*, 2002; Ma and Peterson, 2003). In some plants the development of casparian bands is followed by an additional deposition of hydrophobic substances along the radial walls forming suberin

lamellas. These structures further contribute to the termination of the apoplastic movement (Enstone *et al.*, 2003). In plants exhibiting high salt avoidance, casparian bands and suberin lamellae can also be deposited on walls of hypodermal cells, leading to formation of the exodermis (Perumalla *et al.*, 1990). Unlike endodermis, where formation of casparian bands and suberin lamellas are two distinct processes, formation of these two structures occurs simultaneously during the maturation of the exodermis (Hose *et al.*, 2001; Ma and Peterson, 2001; Enstone *et al.*, 2003; Ma and Peterson, 2003). Also unlike endodermal cells, the exodermis is characterized by a reduced deposition of suberin and lignin (Schreiber *et al.*, 1999; Zeier *et al.*, 1999). Independent studies have unequivocally shown that formation of casparian bands and suberin lamellas only affect free radial movement of ions, like Ca^{2+}, which normally move through the apoplast. The movement of ions like K^{+}, PO_4^{3-} characterized by a symplastic transport are not affected (Russell and Clarkson, 1975; Enstone *et al.*, 2003). Furthermore, besides regulating xylem-loading casparian bands and suberin lamellas also limit ion leakage from the stele back to the cortical cells and soil (Peterson *et al.*, 1993). Unlike the exodermis, the endodermis can better control ions and water intake (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003).

Based on their anatomical characteristics, two types of exodermis are found in plants: uniform exodermis and dimorphic exodermis (Peterson and Enstone, 1996; Enstone *et al.*, 2003; Ma and Peterson, 2003). While a uniform exodermis is composed of a single type of cells developing both suberin lamellas and casparian bands (Enstone and Peterson, 1997), a dimorphic exodermis consists of two cell types: long cells with both casparian bands and suberin lamella, and short cells with only casparian bands (von Guttenberg, 1968; Walker *et al.*, 1984; Ma and Peterson, 2001). These short cells, which act as "passage cells" facilitate the movement of water

and ions into the stele (von Guttenberg, 1968). Depending on the number of cell layers the exodermis can be uni-serrated, (one layer of cells) (Perumalla *et al.*, 1990; Miyamoto *et al.*, 2001; Enstone *et al.*, 2003; Ranathunge *et al.*, 2003) or multi-serrated (multiple layers of cells), (Seago *et al.*, 1999; Soukup *et al.*, 2002; Meyer *et al.*, 2009).

In both root endodermis and exodermis, salinity induces the formation of casparian bands and suberin lamellas closer to the root tip (Shannon *et al.*, 1994). This has been demonstrated in a variety of systems including citron (Walker *et al.*, 1984), cotton (Reinhardt and Rost, 1995), maize (Karahara *et al.*, 2004) and rice (Krishnamurthy *et al.*, 2009). Besides their location, salinity also affect their size. In maize plants grown in 200 mM NaCl the width of both casparian bands and suberin lamellas increases 2-3 folds (Poljakoff-Mayber, 1975; Tester and Davenport, 2003; Kronzucker and Britto, 2011).

Environmental conditions also affect endodermis and exodermis development. Deposition of suberin lamellas is accelerated in maize roots grown in aeroponics, vermiculite or in anaerobic hydroponic solutions (Enstone and Peterson, 2005). Furthermore, relative to plants grown hydroponically, formation of casparian bands and suberin lamellae of plants grown in solid media occurs closer to the root tip (Meyer *et al.*, 2009). These observations reinforce the idea that root modifications in response to salinity are highly flexible, dynamic, and influenced by other environmental factors.

Understanding and improving salt tolerance, in conjunction with the development of effective propagation methods for species like Bur oak, would be highly valuable to enrich tree diversity in urban areas.

2.6 Propagation of Bur oak

Bur oak is mainly propagated directly from seeds (acorns), or through grafting of scions on rootstocks well-adapted to local soils. As a cross-pollinated species, Bur oak trees are highly heterozygous (Dow and Ashley, 1998; Craft *et al.*, 2002; Dutech *et al.*, 2005), an undesirable characteristic due to the genetic inheritance of many responses to biotic and abiotic factors (Steiner, 1995; Hertel and Zaspel, 1996). Even seedlings derived from seeds of the same parent can exhibit contrasting responses. Salt tolerance, like other types of resistance to biotic factors, is controlled by several genes (Vinocur and Altman, 2005), and therefore its inheritance cannot be ensured in cross-pollinated species. To overcome this problem and produce plants with uniform genotypes, vegetative propagation is often employed (Hartmann *et al*., 1997).

As previously mentioned, the most common vegetative propagation method applied to Bur oak is grafting. This method however, is very tedious, time consuming and has a low success rate (Hartmann *et al*., 1997; Sumrah *et al.*, 2002; Sutanto, 2010). Alternative and more efficient vegetative propagation methods, such as propagation via cuttings, need to be optimized.

2.6.1 Propagation via stem cuttings

Propagation from cuttings is very common within the nursery industry. Although any plant material such as leaves, shoots or roots has the potential to be used as cuttings (Hartmann *et al*., 1997; Acquaah, 2005), stem cuttings are commonly used in woody plants (Dirr and Heuser, 1987). Propagation via stem cuttings eliminates the major problem encountered during grafting: graft incompatibility (Hartmann *et al*., 1997). Based on the types of plant and time of cutting collection, stem cutting is classified as softwood (plum and rose), semi-hardwood (magnolia and rhododendron) or hardwood cutting (grapes and willow) (Acquaah, 2005).

Successful propagation via cuttings relies on the genotype used, the age of the donor plant, time of cutting collection, number of leaves on cutting, endogenous hormonal levels and many environmental factors (Middleton *et al*., 1980; Tousignant *et al*., 2003; Acquaah, 2005; Rosier *et al*., 2005).

2.6.1.1 Age of donor plant

The age of the donor plant from which the cuttings are harvested affects rooting (Greenwood and Hutchison, 1993; Rosier *et al.*, 2005). Richer *et al*. (2003) found a negative correlation between rooting potential of *Acer saccharum* cuttings and age of donor plant. Similar results were also reported in *Citrus trifoliata* (trifoliate orange) where cuttings from juvenile trees were more prone to form roots (Bhusal *et al.* 2003). Accumulation of root-inhibitory compounds in old plants is thought to be the reason for these observations (Paton *et al*., 1970; Dirr and Heuser, 1987).

2.6.1.2 Time of cutting collection

Successful propagation via cuttings also depends on collection time (Tousignant *et al*., 2003). For example, rooting percentage of crab apple and apple cuttings was significantly higher if collection occurred in May and July respectively (Burd and Dirr, 1977; Chapman and Hoover, 1981). Cuttings collected after or before these months resulted in substantially lower rooting percentages. The effect of the collection time is mainly associated to the physiological status of the cutting (Nanda and Anand, 1970; Dirr and Heuser, 1987; Acquaah, 2005).

2.6.1.3 Hormone application

External applications of hormones, such as auxins, cytokinins or gibberellins, are often used to enhance rooting (Dirr and Heuser, 1987; Alegre *et al*., 1998). Indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) are the two major auxins stimulating root formation in woody species (Acquaah, 2005). However, rather than a single hormone, combinations of different hormones are often more inductive to rooting (Fogaca and Fett-Neto, 2005; Kesari *et al.*, 2009; Vakouftsis *et al*., 2009). Compared to NAA, the use of IBA is preferred due to its lower toxicity (Al-Salem and Karam, 2001; Kesari *et al.*, 2009). For example, Fogaca and Fett-Neto (2005) reported a higher root formation frequency in both *Eucalyptus saligna* and *Eucalyptus globulus* cuttings treated with IBA, as compared to cuttings treated with same concentrations of NAA. A similar result was also documented in *Pongamia pinnata* (Kesari *et al.*, 2009).

The formation of roots from cuttings is very sensitive to the concentration of hormone utilized. For instance, using different IBA levels (20, 40, 60 and 80 µg/cutting), Aminah *et al*., (1995) observed large differences in rooting responses of cuttings collected from *Shorea leprosula*. Examples of different tissue sensitivity to hormones were also documented by other studies (Ofori, 1996).

2.6.1.4 Environmental factors

The environment can affect the physiological status of the donor plant and influence the production of root-inhibitory compounds (Richer *et al*., 2003; Acquaah, 2005). For example, growth of *Populus nigra* in harsh climate conditions reduced the formation of roots from cuttings. This was ascribed to a suppression of hydrolyzing enzymes converting starch into

essential sugars (Nanda and Anand, 1970). Low rooting was also observed if applications of hormones to cuttings occurred during unfavorable climatic conditions (Nanda *et al.*, 1968).

Other environamental conditions including suitable rooting media, humidity, and temperature can also influence rooting. For instance, a suitable rooting media will ensure proper moisture, enable gas exchange, and limit penetration of light (Hartmann *et al*., 1997). Although perlite/peat mixture is the most common rooting medium, sand has often been used as an alternative medium (Dirr and Heuser, 1987). Humidity also plays a key role during rooting and the use of intermittent misting systems has produced good results (Sutanto, 2010).

2.6.1.5 Leaves

Presence of leaves on the cutting is essential for providing soluble sugars to the growing roots (Nanda and Anand, 1970). The importance of leaves in root formation was demonstrated in *Phaseolus aureus,* where exogenous IBA applications promoted rooting only if leaves were not removed from the cuttings (Middleton *et al*., 1980). These results were similar to those reported in *Acer rubrum* where success of rooting correlated to the number of leaves on the cuttings (Dirr and Heuser, 1987).

2.6.2 Root pruning and transplantation

Low transplantation success of both nursery-grown and field-grown Bur oak plants is a major problem limiting the relocation of this species into new areas (Allen and Kuta, 1994). This undesirable characteristic is due to presence of a single tap root which can be easily damaged during transplantation (Weaver and Kramer, 1932). Retention of a functional and vigorous root system is indeed paramount when transplanting trees. In many instances harvesting the entire or

the majority of the root system is a difficult task, as documented in several species (Gilman *et al*. 1987; Watson and Sydnor, 1987). Root damage in transplanted plants can significantly reduce their performance and ability to re-establish in new and often diverse environments (Nussbaum, 1969; Preisig *et al*., 1979; Nichols and Aim, 1983). Transplantation success is generally greater in plants with fast growing roots and with short root ball systems composed of many lateral roots (Lyr and Hoffmann 1967; Watson and Himelick, 1982b). These desirable characteristics are obtained through root pruning (Solfjeld and Hansen, 2004; Watson, 1986; Ruehle and Kormanik, 1986).

Root pruning has been reported to improve root structure and transplantation in a number of plant species (Solfjeld and Hansen, 2004). Independent studies have shown that production of auxins at the pruned root end aids the transport and re-allocation of nutrients within the root tissue improving growth (Watson and Himelick, 1982b; Watson and Himelick, 1983; Solfjeld and Hansen, 2004). Root pruning in apple, pine, and green ash significantly enhanced formation of new roots (McDonald *et al*., 1984; Arnold and Strove, 1989b; Arnold and Young, 1991).

Plants subjected to root pruning show enhanced water use efficiency due to a general reduction in shoot growth accompanied by increased stomatal conductance (Stupendick and Shephered, 1980; Arnold, 1987; Arnold and Struve, 1989b). Reduction in transpiration rate is paramount for successful transplantation. Transplanted plants in fact often experience damages due to water loss (Broschat and Donselman, 1986). Besides enhancing water management, the use of root pruning containers also controls root-girdling, a major problem for plants grown in conventional root trays (Appleton, 1993; Marler and Willis, 1996).

In conclusion, examining the performance of oak species to high salt conditions and conducting preliminary studies on vegetative propagation via cuttings are important steps for the

introduction of oaks into urban environment. These issues are addressed in this thesis. The first chapter investigates the effects of increasing NaCl levels on morphological and physiological characteristics of Bur and Red oak seedlings, the second chapter examines the role of age of donor plant, effect of exogenous hormonal application, age of explant and date of collection on propagation via cuttings.

3. Chapter 1: Response of Bur and Red oak to NaCl-induced salinity

A modified version of this chapter has been published in:

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3.1 Introduction

Forest management practices in urban plantations are leading to extensive monocultures reducing tree diversity, increasing the possibilities of disease and pest outbreak and threating entire ecosystems (Kouki, 1994). For example, a spruce monoculture caused by improper plantation strategies in Europe has contributed to the outbreak of spruce bark beetles (*Ips typographus*) (Grégoire, 1988). Similarly, the low-diversity forests of North America, dominated by ash species, have facilitated the rapid spread of Emerald Ash Borer causing destruction of millions of ash genus trees (Ronald, 2006). On the other hand, more diverse tree stands can provide additional security against diseases and pests (Jactel *et al*., 2005). The dominance of ash trees in Manitoba makes our forests very susceptible to pest or disease outbreaks (Ronald, 2006). Therefore, increasing tree diversity in human-managed forests is essentials for the sustainability of urban forests.

Bur oak (*Q. macrocarpa)* and Red oak (*Q. rubra*) are native to North American and thrive in cold and dry environments (Allen and Kuta, 1994). High drought and cold tolerance provided by deep root systems give advantages to these species over others in the same area (Gale and Grigal, 1987). Due to these characteristics, oak species are good candidates to be reintroduced in urban forests in order to increase tree diversity in these ecosystems. Before

introducing these hardy species into urban environments, their responses to salinity must be assessed. Salinity in urban areas, especially in Canada, is mainly due to salts (especially NaCl) used for winter road de-icing (Transportation Research Board, 1991; Environment Canada, 2001). When ice melts, runoff waters deposit up to 90% of this salt within 13m of the roads, severely affecting the roadside plants (McBean and Al-Nassri, 1987; Viskari and Kärenlampi, 2000).

Soil salinity has been one of the major threats to agriculture worldwide, significantly reducing the production on major food crops and economical plants (Croser *et al*., 2001). In North America alone, almost 800 million ha of agriculture land is currently affected by salinity (Rengasamy, 2006). In plants, high salt concentration interferes with basic metabolism, alters anatomy and morphology, reduces growth and often leads to death (Munns, 2002; Zhu, 2007). A significant number of studies have examined plant tolerance and susceptibility to salt stress with the ultimate goal to develop plants capable of withstanding high salt levels.

Salt stress in plants is often the consequence of elevated levels of Na^+ and Cl^- ions (Gadallah, 1999; Parida *et al*., 2004), with symptoms varying greatly depending on the developmental stage of the plant, degree of salinity, and duration of salt exposure (Bernstein and Kafkafi, 2002; Munns and Tester, 2008). Besides disrupting water and mineral balance, salinity causes ion toxicity, and oxidative stress (Munns, 2002; Zhu, 2007). One of the first symptoms experienced by plant cells exposed to high salt levels is the lowering of the water potential which compromises water uptake and the execution of metabolic and physiological processes, including cell division and elongation (Cheeseman, 1988; Allakhverdiev *et al*., 2000; Flowers, 2004; Carillo *et al*., 2011; Osakabe *et al*., 2014). If prolonged, salinity can also result in the undesirable accumulation of toxic ions within the cells and their movement through the

transpiration stream causing leaf necrosis, premature senescence of leaves, and reduction in photosynthesis (Hasegawa *et al*., 2000; Khan, 2001; Munns, 2002; Abogadallah, 2010). Mineral imbalance is also experienced, as Na^+ and Cl^- compete with the uptake of other ions essential for normal development (Gabr, 1999; Khan *et al*., 2000; Hu and Schmidhalter, 2005). Deficiency of these essential nutrients alters the functionality of plasma membranes and reduces enzymatic activity (Tester and Davenport, 2003; Munns and Tester, 2008), leading to limited ATP production, reduced gas exchange, and abnormal protein synthesis (Brady *et al*., 1984; Alam, 1999; Mansour, 2000).

Reactive oxygen species (ROS) like superoxide radicals ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen molecules ($^1O_2$) also accumulate under high salinity conditions (Tuteja, 2007) resulting in damages to several cellular components (Vinocur and Altman, 2005). Elevation in ROS is often due to their increased biosynthesis, or a depression of the antioxidant machinery composed by several ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidise (APX), catalase (CAT), and glutathione reductase (GR) (Allan and Fluhr, 1997; Foyer and Noctor, 2003; Vinocur and Altman, 2005). Transgenic plants over-expressing antioxidant enzymes are more tolerant to salinity (Lee *et al*., 2001; Mittova *et al*., 2003; Tuteja, 2007), and plants with higher activities of these enzymes generally perform better under high salt levels (Rodriguez-Rosales *et al*., 1999; Rios-Gonzalez *et al*., 2002).

Plants have developed a variety of strategies to cope with salinity. These include a reduction in leaf area, adjustment in stomata conductance, increase in chloroplast number, sequestration of toxic ions in vacuoles, production of solutes to compensate for the lowered water potential of the soil, and structural modifications to impede movements of toxic ions (Khan *et al*., 1995; Zhifang and Loescher, 2003; Zhu, 2003). Formation of casparian bands and

suberin lamellas in root endodermis and exodermis are common strategies to reduce ion movement under salinity (Zimmermann *et al*., 2000; Bucking *et al*., 2002; Enstone *et al*., 2003). These root modifications have also been associated with increased salt tolerance in a number of plant species (Karahara *et al*., 2004; Krishnamurthy *et al*., 2009; Kronzucker and Britto, 2011).

The objective of this study is to evaluate the degree of salt tolerance in Bur and Red oak seedlings grown in increasing levels of NaCl. Root growth and anatomy, leaf injury, nutrient uptake and activity of antioxidant enzymes were used as parameters to evaluate plant performance.

3.2 Materials and methods

Acorns of Bur oak were collected from single mature healthy Bur oak tree growing at the Riverbend Orchards Inc., Portage La Prairie, MB, Canada, while all acorns of Red oak were collected from Eastern Iowa, USA. These acorns were first planted in a mixture of sand:soil:peat (2:1:1, V/V) and subsequently stratified at 4°C for 3 months to achieve uniform germination. The stratified acorns were then transferred to the greenhouse at the University of Manitoba, Winnipeg, Canada (22 ± 5°C and 16h daylight) and watered regularly. One week after emergence, the seedlings were randomly selected and transferred to plastic containers filled with ½ strength Hoagland's solution (Renault *et al*., 2001). These containers were then transferred into growth chambers with a 22°C day (16h)/ 18°C night (8h) photoperiod, 70% relative humidity, and 400 μmol m^{-2} s^{-1} light intensity, deemed best for optimal plant growth (Davis *et al.*, 2014). Seedlings were held firmly using styrofoam plugs in holes made through the container lids, and air was bubbled through into the rooting media using irrigation pumps and perforated irrigation tubes (Supplement fig. 1). After acclimatization for 1 week in the same nutritional

media, the seedlings were exposed to four different salt treatments (0, 25, 50 or 75 mM NaCl). Desired concentrations of salt, in high NaCl treatments, were reached by 25 mM increments of NaCl every 12 hr to avoid shock injuries caused by sudden exposure to salt. Nutrient media in each container were replaced once during the first week of acclimatization, and twice weekly during the subsequent weeks in order to avoid algal growth. Three biological replicates, each consisting of 10 plants, were used for Bur and Red oak at each treatment to limit experimental errors and improve efficiency of statistical testing. All analyses were conducted after 1, 2 and 3 weeks of salt treatments, unless specified otherwise.

3.2.1 Morphological parameters and nutrient analysis

The effect of NaCl on root growth was analyzed by measuring the root length of each plant every 7 days during the 3 weeks of salt treatments. Leaf injury was estimated using a visual 0-5 numerical scale where 0 indicates no injury while 1, 2, 3, 4 and 5 corresponds to 1-25%, 26–49%, 50–75%, 76–99% and 100% leaf injury respectively (Campbell *et al*., 2015). Fresh and dry weights were measured after 3 weeks of salt treatments. Leaf and root tissues were harvested separately, washed with distilled water to remove debris, and weighted tissue were dried using a 60°C oven. Dried tissue samples were also utilized for macro- and micro-nutrient analysis performed by Stratford Agri Analysis Laboratories (Stratford, ON, Canada) following the procedure available at http://www.stratfordagri.ca/.

3.2.2 Antioxidant enzyme activity

Activity of the major antioxidant enzymes was measured in leaf tissue of both Bur and Red oak seedlings after 3 weeks of salt treatments. For catalases (CAT) and total peroxidase (POD)

activities, tissue (0.5g) was first frozen in liquid nitrogen and then homogenized in 6 ml of ice-cold extraction buffer (50 mM NaH_2PO_4/Na_2HPO_4 (pH 7.0), 0.2 mM EDTA and 1% (w/v) polyvinylpyrrolidone) in an ice-cold mortar and pestle. The homogenate was filtered through cheesecloth followed by centrifugation at 15,000g for 20 min at 4°C. The extract was then utilized to measure the enzymatic activity as described by Zhang and Kirkham (1995).

Catalase activity was monitored by following the decline in absorbance caused by the decomposition of H_2O_2 for 1 min at 240 nm (ε = 39.4 M^{-1} cm^{-1}, Nelson and Kiesow, 1972) in a 3ml reaction containing 50 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.0), 15 mM H_2O_2, and 20 µl enzyme. The tissue extract was added last to initiate the reaction.

Total peroxidase activity was measured by following the oxidation of guaiacol at 470 nm (ε = 26.6 mM^{-1} cm^{-1}, Chance and Maehly, 1955) for 1 min in a 3ml reaction mixture containing 2.83 ml of 10 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.0), 50 µl of 20 mM guaiacol, 0.1 ml tissue extract and 20µl of 40 mM H_2O_2.

For ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), 8 g of leaf tissue was homogenized, using sea sand, in 10 ml extraction buffer (100 mM NaH_2PO_4 /Na_2HPO_4 pH 7.8, 2% Triton X-100, 5 mM ascorbate and 400 mg insoluble polyvinylpolypyrrolidone) for 2-3 minutes. The resulting mixture was incubated on ice for 30 min and then centrifuged at 20,000g for 30 min at 4°C. The supernatant was further purified using a Sephadex G-25 column equilibrated with 100 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.0). For the APX determination 1 mM ascorbate was included in the equilibration buffer (Schwanz and Polle, 2001). Methods described by Zhang and Kirkham (1995) with slight modifications were used to measure APX, GR and DHAR activity.

The activity of APX was measured by following the decline in absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}, Nakano and Asada, 1981) for 1 min. The reaction mixture consisted of 0.5 mM ascorbic acid, 0.1 mM EDTA, 50 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.0), 0.1 mM H_2O_2 and 100 µl tissue extract.

The activity of GR was measured by recording the oxidation of NADPH at 340 nm (ε = 6.2 mM^{-1} cm^{-1}, Cakmak *et al*., 1993) for 1 min in a reaction mixture composed of 50 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.8), 0.1 mM EDTA, 0.2 mM GSSG, 0.2 mM NADPH, and 150 µl of tissue extract. Oxidized glutathione (GSSG) was added to the reaction mixture to initiate the oxidation of NADPH.

The activity of DHAR was measured by following the increase in absorbance at 265 nm (ε = 14 mM^{-1} cm^{-1}), due to the production of ascorbic acid, for 1 min. The reaction mixture consisted of 50 mM NaH_2PO_4 /Na_2HPO_4 (pH 7.0), 2.5 mM GSH, 0.1 mM EDTA, 0.2 mM dehydroascorbic acid and 100 µl of tissue extract (Cakmak *et al*., 1993).

Protein content in the tissue extracts was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard protein. An Ultrospec 2100 pro UV/visible spectrometer was used for all measurements.

3.2.3 Root sectioning and microscopy

After 3 weeks of salt treatments, free-hand and microtome sections from root tissue were carefully examined to visualize salt-induced structural modifications. Free-hand transverse sections were generated along the profile of the root at 1, 5, 10, 15, 20, 25, and 30 cm from the tip (Peterson *et al*., 2008). The sections were immediately placed in water to avoid dehydration and their anatomical features were studied with selected stains using section holders

(Supplement fig. 2). Casparian bands were visualized by staining with 0.1% (w/v) berberine hemisulphate for 15 min followed by counterstain with 0.5% (w/v) aniline blue for 30 min (Brundrett *et al*., 1988). The sections were immedietly mounted in 0.1% (w/v) ferric chloride ($FeCl_3$) solution containing 50% glycerol (Brundrett *et al*., 1988) and analyzed under UV light (excitation filter 330–380 nm, dichroic mirror 400 nm, and a barrier filter 420 nm) using a Leica DMRE microscope (Leica Microsystems Inc.).

Suberin was stained with a 0.1% Sudan red 7B solution containing 50% polyethylene glycol for 1 h (Brundrett *et al*., 1991), while for lignin visualization root sections were mounted directly in the 20% solution phloroglucinol-HCl solution and examined immediately under white light (Jensen, 1962).

To further study root developmental at a higher resolution, 1 cm root segments, dissected at 1, 5, 10, 15, 20, 25 and 30 cm from the tip were fixed, sectioned and stained for general histology. The root tissue was first fixed with 1.6% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 6.9) for 24 h (Yeung and Saxena, 2005), and then dehydrated by one change of 2-methoxyethanol and two changes of absolute ethanol at 24 h intervals. The dehydrated tissue was embedded in historesin (Yeung, 1999; Stasolla *et al*., 2004; Yeung and Saxena, 2005) and sectioned (3μm) using a Leica RM2145 autocut rotary microtome. The sections were allowed to dry overnight at 37°C before being stained for general histological analysis with 0.1% toluidine blue in 0.1 M benzoate buffer (pH 4.4) for 45s. Toluidine blue stains carbohydrates (pink), and protein and lignin (blue) (O'Brien and McCully, 1981; Peterson *et al*., 2008).

3.2.4 Statistical analysis

Experiments were performed using a completely randomized design with three biological replicates, each including at least 10 plants. For each test, representative plants were randomly chosen from a larger group to minimize any experimental bias. The collected data were analyzed by two-way ANOVA with the SPSS program (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. IBM Corp., Armonk, NY, USA). Differences between various parameters were tested by comparing treatment means from ANOVA test using Duncan's multiple range test (a = 0.05).

3.3 Results

3.3.1 Effect of salinity on oak growth and morphology

The effects of four different NaCl concentrations were carefully evaluated at weekly intervals for 3 weeks for various morphological parameters of Bur and Red oak seedlings. Increasing levels of salt reduced root growth in both species with the most significant differences observed at 50 mM and 75 mM NaCl (Fig. 1a). Relative to control (0 mM NaCl), root growth after 3 weeks in 75 mM NaCl was reduced by 40% in Bur oak, while almost ceasing in Red oak roots exposed to the same salt level (Fig. 1a). A similar trend was also apparent at 50mM NaCl with a reduction in growth of about 30% for Bur oak and 70% for Red oak. Morphological analysis revealed that at the higher salt levels (50 mM and 75 mM NaCl) the root tips of Red oak became brown and started dying, while those of Bur oak remained alive (Fig. 1b). Salinity also reduced root branching, especially in Red oak (Fig. 1b), and decreased both fresh and dry weight of roots (Fig. 2). Exposure for three weeks to different levels of NaCl, increased sodium (Na) accumulation in both root and leaf tissues (Fig. 3). Relative to Bur oak, Na^+ intake was more

pronounced in Red oak with statistically significant differences observed at 50 mM and 75 mM for roots and 25 mM and 50 mM for leaves. Of interest, no differences in Na^+ levels were observed in leaves of plants grown in 75 mM NaCl (Fig. 3).

Positive correlations exist between salt concentration and leaf injury (Ferguson and Grattan, 2005). Leaf injury, estimated using an arbitrary scale (see "Materials and methods" for details), increased in both species subjected to salinity reaching maximum values with 75 mM NaCl (Fig. 4). Differences between species were observed at lower salt levels (25 mM); compared to Bur oak, Red oak seedlings showed significant signs of injury after only one week (Fig. 4).

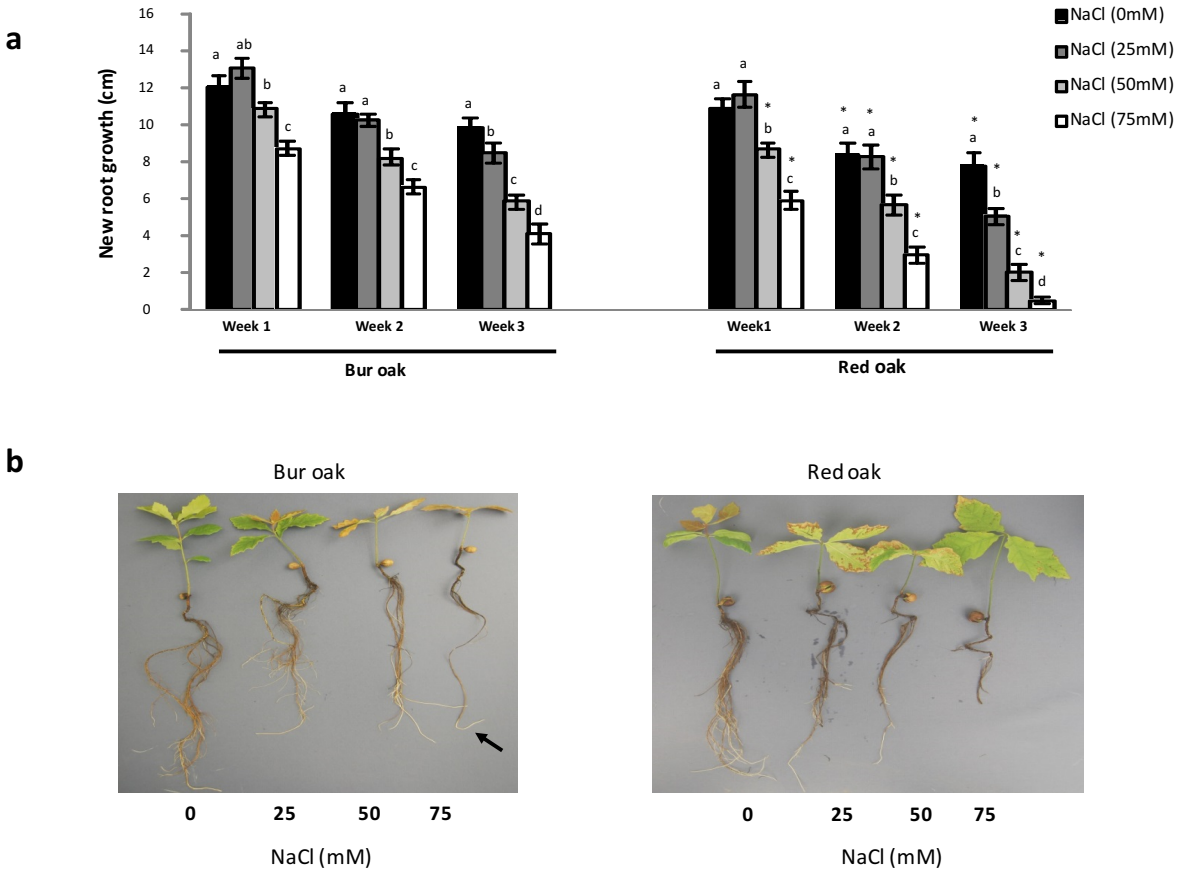


Fig. 1 **(a)** Weekly root growth of Bur and Red oak seedlings exposed to 0, 25, 50 and 75 mM NaCl. Values ± SE are means of three biological replicates. Letters on bars indicate statistically significant values ($p < 0.05$) within each species from their respective control value (0 mM NaCl) at the same week. Asterisk indicates statistically significant values ($p < 0.05$) of Red oak, relative to Bur oak, at the same salt concentration and week.
(b) Seedling morphology of Bur and Red oak grown for 3 weeks in the presence of four different concentrations of NaCl.

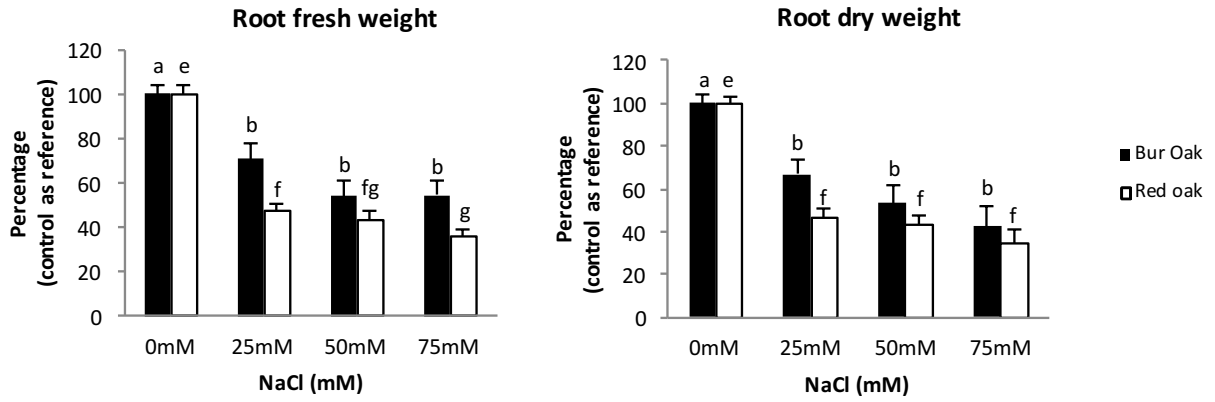


Fig. 2 Fresh and dry weight of Bur and Red oak roots after 3 weeks of growth in 0, 25, 50 and 75 mM NaCl. Values ± SE are means of three biological replicates. Alphabetical letters on bars indicate statistically significant values ($p < 0.05$) within each species from their respective control (0 mM NaCl).

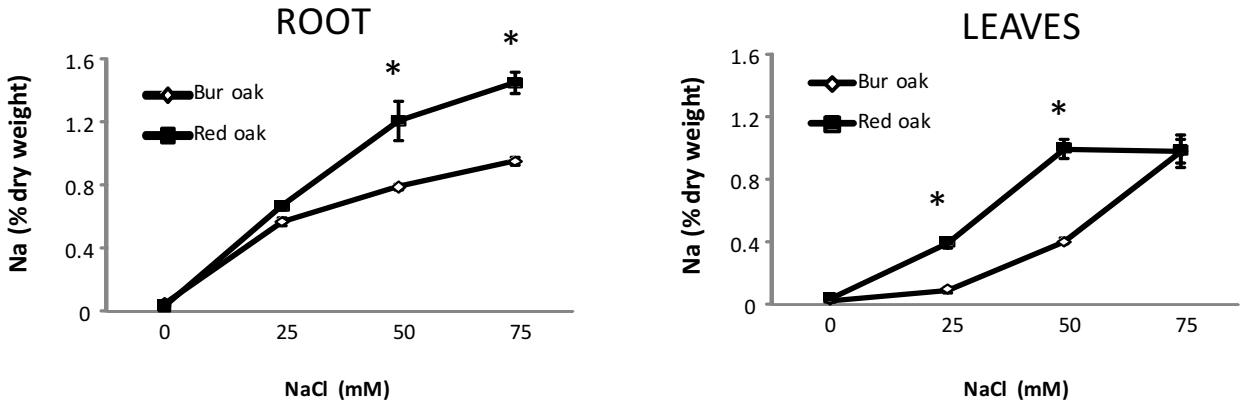


Fig. 3 Sodium (Na) concentration in root and leaf tissue of Bur and Red oak seedlings grown in the presence of 0, 25, 50 and 75 mM NaCl. Values ± SE are means of three biological replicates. Asterisk indicates statistically significant differences ($p < 0.05$) between species at the same salt concentration.

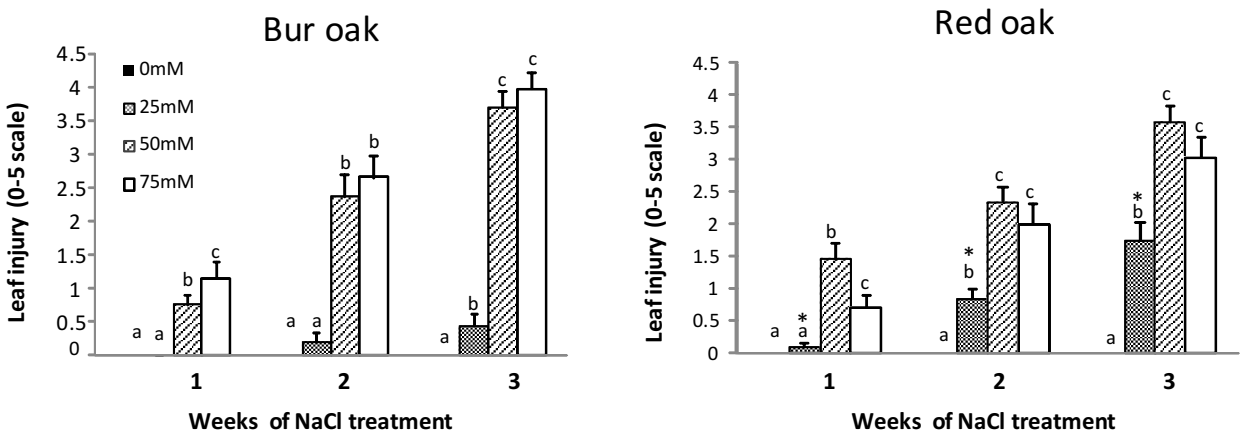


Fig. 4 Leaf injury in Bur and Red oaks seedlings after 1, 2 and 3 weeks of exposure 0, 25, 50 and 75 mM NaCl. Numerical scale used to quantify leaf injury was: 0 = no injury,1 = 1-25% injury, 2 = 25–49% injury, 3 = 50–75% injury, 4 = 76–99 % injury and 5 =100 % injury. Values ± SE are means of three biological replicates. Letters on bars indicate statistically significant values ($p < 0.05$) within each species from their respective control value (0 mM NaCl) at the same week. Asterisk indicate statistically significant values ($p < 0.05$) of Red oak, relative to Bur oak, at the same salt concentration and week.

3.3.2 Effect of salinity on antioxidant enzyme activity

The correlation between excessive production of reactive oxygen species (ROS) levels and cellular damage in plants exposed to salinity (Zhu, 2001; Wang *et al*., 2008) prompted a study on antioxidant response mechanisms. Synthesis of antioxidants and activation of antioxidant enzymes are effective strategies employed by plants to reduce ROS production under different types of stress (Mittova *et al*., 2003; Abogadallah, 2010). These strategies increase the chances of survival against the deleterious effects of superoxide anions ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). The initial line of defense against ROS involves the enzymes superoxide dismutase (SOD), which scavenges superoxide anions, as well as catalases (CAT), peroxidases (POD), and ascorbate peroxidases (APX) which remove hydrogen peroxide (H_2O_2) (Fig. 5a). This last enzyme, APX, found in both cytoplasm and chloroplasts (Foyer and Noctor,

2011) requires ascorbic acid produced *de novo* or through the reduction of dehydroascorbate mediated by dehydroascorbate reductase (DHAR) in the glutathione-ascorbate cycle. This cycle is then completed by the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) catalysed by glutathione reductase (GR) (Polle, 2001) (Fig. 5a).

The activities of CAT, SOD, and DHAR in leaf tissue of Bur oak did not show apparent fluctuations at any salt concentration (Fig. 5b). Lower activities of the three enzymes were measured in Red oak compared to Bur oak. The activities of POD and GR showed similar trends in both species with a gradual increase with elevated levels of NaCl. Compared to Bur oak, however, lower activity levels were observed in Red oak for POD and GR at almost all salt levels. Relative to Bur oak, a higher activity of APX was measured in Red oak grown in the presence of 25 mM and 75 mM NaCl (Fig. 5b).

3.3.3 Macro- and micro-nutrient concentrations in leaf and root tissues of Bur and Red oak at different NaCl levels

High concentration of NaCl severely effects nutrient availability by hindering nutrient uptake and allocation in plant tissues (Gabr, 1999; Khan *et al*., 2000; Hu and Schmidhalter, 2005). Micro- and micro-nutrient levels were measured in leaf and root tissues of the both oak species exposed to different salt levels (Fig. 6). The concentration of magnesium (Mg^{2+}) in Bur oak leaves was not altered by salt, whereas a significant reduction of the same element was observed in Red oak grown with any level of salt. An opposite trend was noted in root tissue, with Bur oak displaying a lower accumulation of Mg^{2+}. Like Mg^{2+}, the level of calcium (Ca^{2+}) in leaf tissue was greater in Bur oak, especially at 50 mM and 75 mM NaCl. In roots, however, this trend was reversed with higher values recorded for Red oak (Fig. 6).

In Bur oak leaves the levels of nitrogen (N) and potassium (K) were not affected by NaCl, while a rise in the levels of both elements was observed in Red oak leaves. Increased salinity decreased the concentration of K^+ in the root tissue of both species.

Salt treatments in Bur oak did not alter the amount of zinc (Zn), which declined in Red oak roots especially at 50 mM NaCl. The amount of copper (Cu) increased in Bur and Red oak leaves exposed to salt. Relative to Bur oak, Red oak roots exhibited higher amounts of Cu at 0, 25, and 75 mM NaCl (Fig. 6).

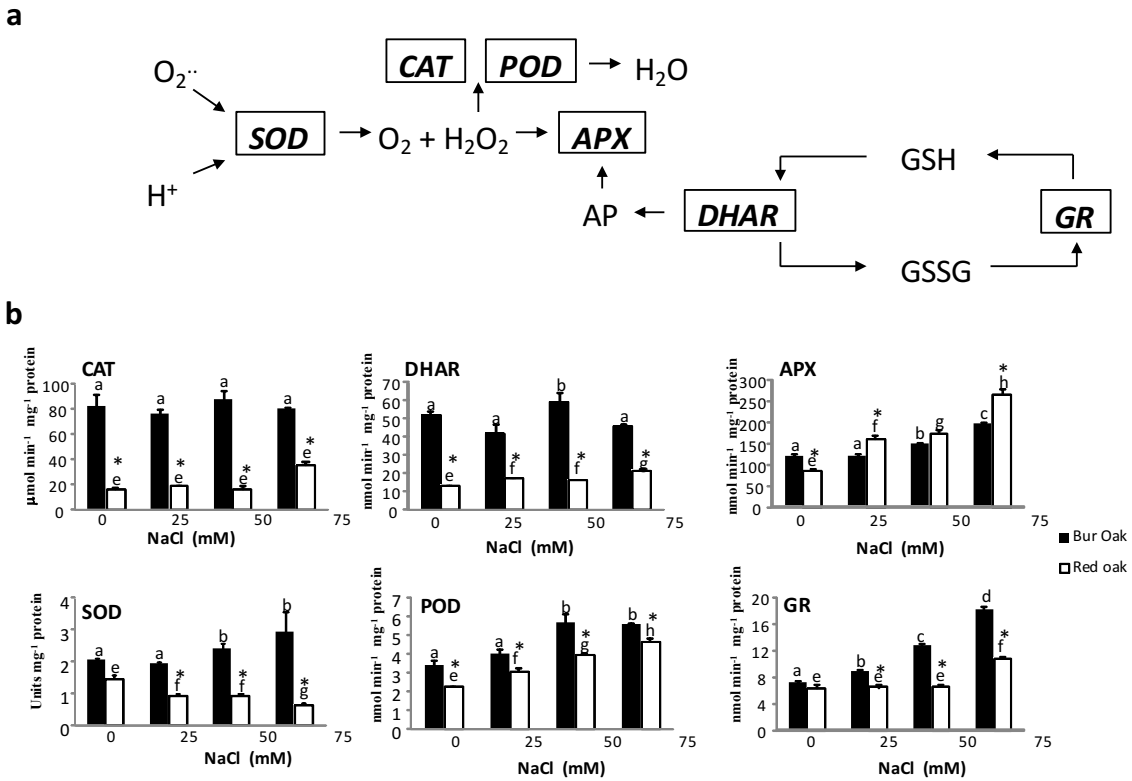
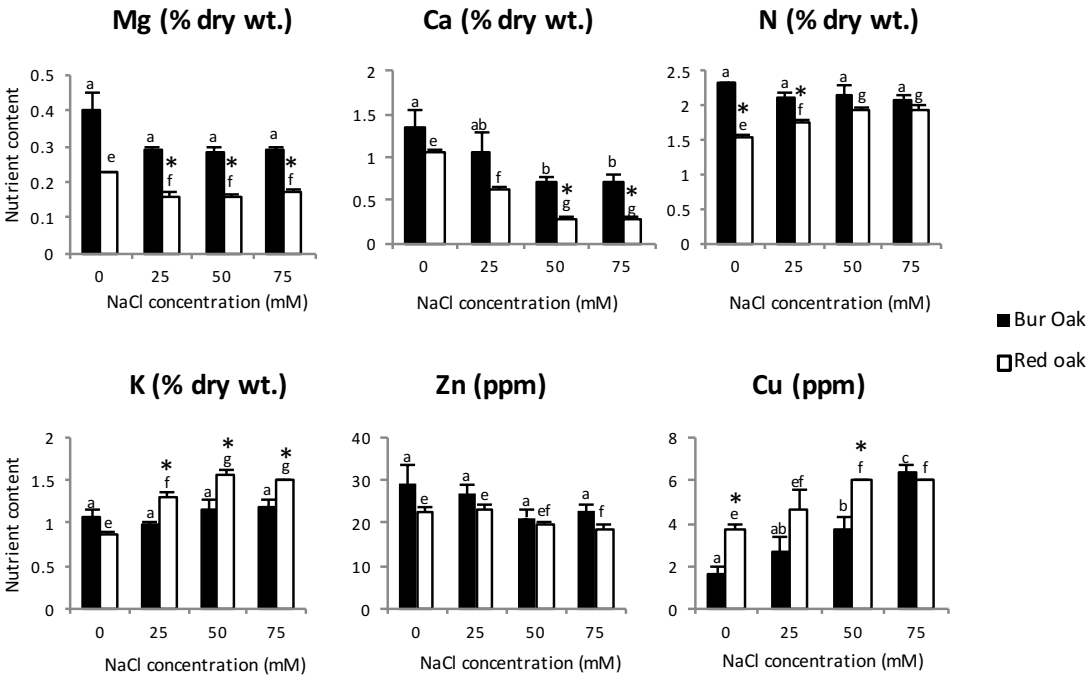


Fig. 5 **(a)** Schematic representation of the antioxidant system operating in plants. SOD = superoxide dismutase, CAT = catalase, POD = total peroxidase, APX = ascorbate peroxidase, AP = ascorbate, DHAR = dehydroascorbate reductase, GR = glutathione reductase, GSH = reduced glutathione, GSSG = oxidized glutathione.
(b) Activities of antioxidant enzymes in leaf tissue of Bur and Red oak seedlings grown for 3 weeks in the presence of 0, 25, 50 and 75 mM NaCl. Values ± SE are means of three biological replicates. Letters on bars indicate statistically significant values ($p < 0.05$) within each species from their respective control (0 mM NaCl). Asterisk indicates statistically significant values ($p < 0.05$) of Red oak relative to Bur oak at the same salt concentration. One unit of SOD activity was defined as the amount of the enzyme required to cause a 50 % inhibition on the rate of nitro blue tetrazolium reduction.

(a) Leaf tissue



(b) Root tissue

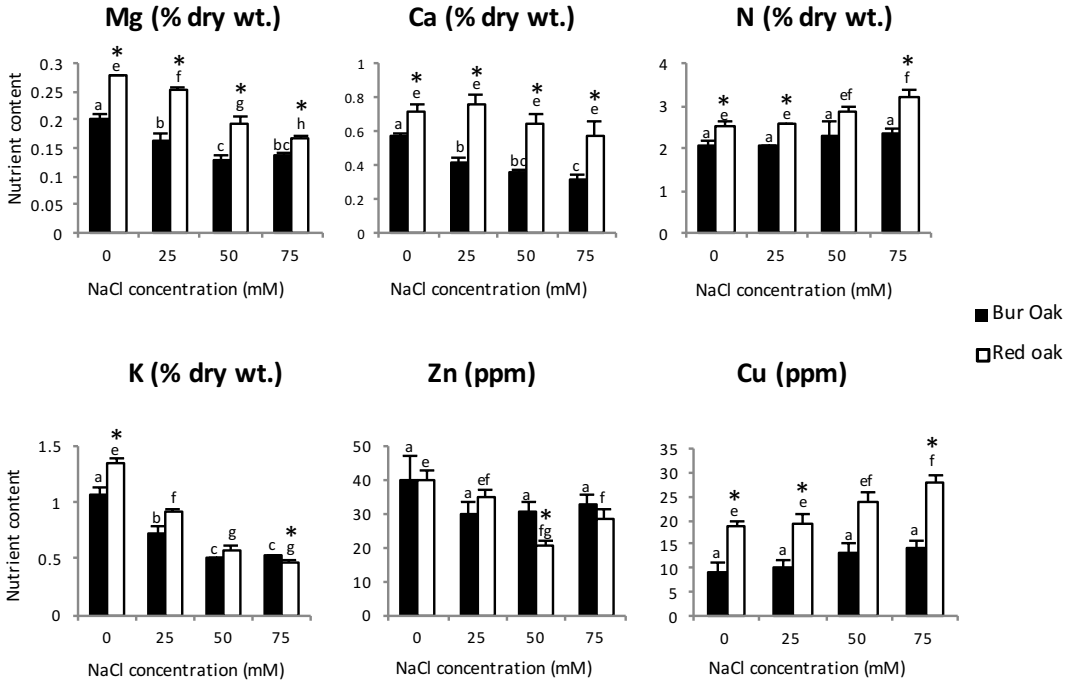


Fig. 6 Levels of macro- and micro-nutrients in leaf **(a)** and root **(b)** tissue of Bur and Red oak after 3 weeks of exposure to 0, 25, 50 and 75 mM NaCl. Values ± SE are means of three biological replicates. Letters on bars indicate statistically significant values ($p < 0.05$) within each species from their respective control value (0 mM NaCl). Asterisk indicates statistically significant values ($p < 0.05$) of Red oak, relative to Bur oak, at the same salt concentration.

3.3.4 Anatomical analysis of Bur and Red oak roots grown at different salt levels

Movement and absorption of nutrients in root tissue is mediated and controlled by structural modifications which include the endodermis and peridermis. The development of both endodermis and peridermis is characterized by the formation of Casparian bands and suberization, which enhance tolerance to salinity by impeding the apoplastic influx of toxic ions (Enstone *et al*., 2003; Ma and Peterson, 2003). In roots of the Bur oak control (0 mM NaCl), Casparian bands, detected with berberine hemisulphate, were first visible in the endodermis at 5 cm from the root tip (Fig. 7a- Row 2). At this distance, almost all endodermal cells had Casparian bands, with the exception of a few (Fig. 7a- Arrow 1). Sudan stain at 5 cm confirmed the presence of a thick suberin layer in many endodermal cells (Fig. 7a- Row 2). At 10 cm from the root tip, anticlinal divisions of the pericycle originated the peridermis (Fig. 7a- Arrow 2), and a heavy signal for Casparian bands and suberin was detected in both endodermis and peridermis (Fig. 7a- Row 3). In older root tissue (15 and 25 cm from the tip) the peridermal cells accumulated inclusions (possibly phenolic compounds) staining blue with Toluidine Blue O and the outer cells separated and eventually died (Fig. 7a- asterisks). Compared to Bur oak, formation of Casparian bands and suberization of the endodermal layer in Red oak occurred in more mature tissue. Suberization of the endodermis started at 5 cm from the tip (Fig. 7b- Arrow 3 & 4) and was only completed at 10 cm (Fig. 7b- Row 3). Anticlinal divisions (Fig. 7b- Arrow

5) originating a peridermal layer were first observed at 25 cm from the tip. Even in mature tissue, the intensity of the berberine hemisulphate UV signal and Sudan red signal was fainter than that observed in Bur oak tissue.

The effect of salinity on root morphology was also investigated by determining the lowest concentration of NaCl sufficient to induce the formation of Casparian strips and suberization of endodermis or peridermis closest to the root tip. In Bur oak, 25 mM NaCl was sufficient to induce the formation of Casparian bands and suberization around the endodermal cells at 1 cm from the root tip, while 50 mM NaCl was necessary for the formation of a mature peridermal layer at 5 cm (Fig. 7c). In Red Oak, similar changes were apparent only with the highest (75 mM) NaCl level which induced suberization of the endodermis at 1 cm from the tip and a fully mature peridermal layer at 10 cm (Fig. 7d).

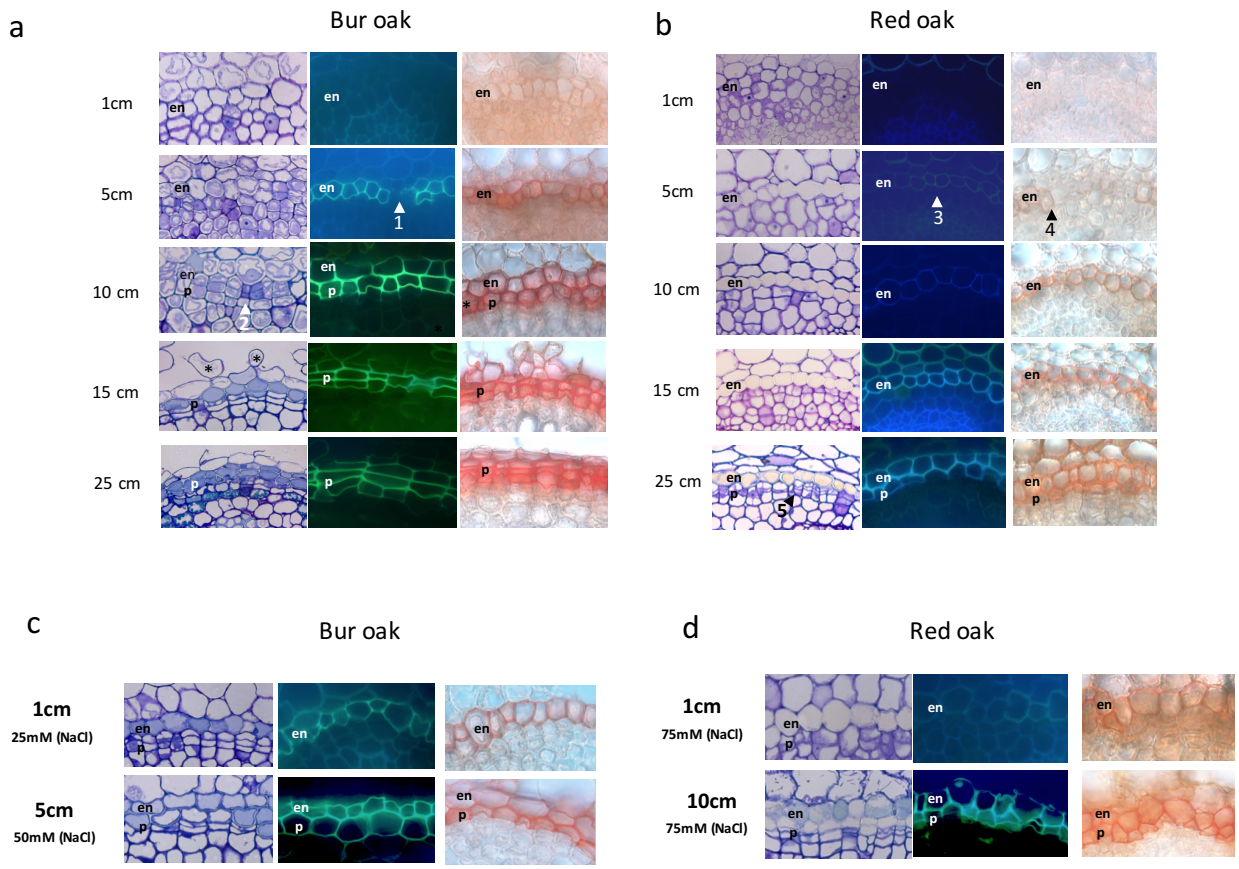


Fig. 7 Structural characteristics in the roots of Bur **(a)** and Red **(b)** oak grown in the absence of NaCl. 1 cm, 5cm 10cm, 15cm and 25 cm indicate distance of sections from the root tip. Visualization of general tissue morphology using Toluidine Blue O (left panels), casparian bands using Berberine hemisulphate (central panels), and suberin using Sudan red (right panels). (en) = endodermis; (p) = periderm; Arrow 1 = absence of casparian bands; Arrow 2 = anticlinal divisions originating the periderm; Arrow 3 = casparian band formation; Arrow 4 = suberin deposition on cell walls; asterisk (*) = cells outside the periderm destined to die.
(c, d) Lowest NaCl concentrations inducing Casparian bands and suberization of endodermis (en) and periderm (p) closest to the root tip of Bur oak **(c)** and Red oak **(d)**.

3.4 Discussion

High NaCl levels had detrimental effects on root growth and shoot development of both Bur and Red oak plants. The negative correlation between root growth and NaCl-induced salinity has been reported previously in several species, including tomato (*S. lycopersicum*) and cotton (*Gossypium spp.*) (Mohammad *et al*., 1998; Meloni *et al*., 2001). Adverse effects of NaCl on root growth are mainly related to both the concentration of the salt and the time of exposure. Immediate reduction in root growth following exposure to NaCl is often considered a defense mechanism which diverts resources from root cells towards "maintenance" and "repairing" processes in photosynthetic tissues (Achard *et al*., 2006; Flowers and Colmer, 2008). Along with the reduction in primary root growth, salinity also decreased root branching, especially in Red oak; a phenotype observed in a number of salt-sensitive plants (glycophytes) when grown under high salt levels (Shannon and Grieve, 1999). The higher salt sensitivity exhibited by Red oak was further substantiated by the presence of necrosis at the root tips (Fig. 1b).

In both species, high salinity levels facilitated the accumulation of Na^+ in leaf tissue causing extensive leaf injuries (Fig. 4), an observation consistent with the degradation of the photosynthetic machinery caused by salt toxicity (Ferguson and Grattan, 2005). Compared to Bur oak however, Red oak grown in the presence of 25 mM NaCl accumulated more Na^+ in leaf tissue and exhibited more pronounced leaf damage (Fig. 3 and 4). At the same level of salinity (25 mM) both species accumulated the same amount of Na^+ in the root (Fig. 3). These observations suggest the existence of mechanisms limiting root-shoot translocation of Na^+ in Bur oak. This strategy, based on a tight control of ion-loading in the vascular tissue has been reported in other species subjected to salinity stress (Davenport *et al*., 2005). A good example is durum wheat (*T. turgidum*), where salt tolerant varieties translocate Na^+ from the root to the shoot at

much slower rate than salt sensitive varieties (Davenport *et al*., 2005). It must be noted, however, that in Bur oak this protective mechanism might not be effective at higher (50 mM and 75 mM) NaCl levels, which induce extensive leaf injuries comparable to those in Red oak (Fig. 4).

Under saline conditions leaf injury is often the result of an over-production of reactive oxygen species (ROS) caused by increased biosynthesis and/or a depression in ROS scavenging mechanisms (Foyer and Noctor, 2003; Tuteja, 2007). If elevated, ROS can induce severe damage to nucleic acids, membranes and physiological processes often resulting from the destabilization of chloroplast and mitochondrial enzymes (Bartoli *et al*., 2004; Vinocur and Altman, 2005). For example, excessive accumulation of superoxide and/or hydrogen peroxide in cell organelles occurs during salt stress conditions compromising photosynthesis and inducing necrosis (Hernandez *et al*., 2001; Foyer and Shigeoka, 2011). A number of studies have reported that an increased activity of the cellular antioxidant machinery associated with increased activities of glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidise (APX), peroxidase (POD) and catalase (CAT) alleviate salinity stress (Meneguzzo and Navarilzzo, 1999; Rios-Gonzalez *et al*. 2002). Better performance under high salt conditions was also observed in plants over-expressing genes encoding for some of these enzymes (Roxas *et al*., 2000; Mittova *et al*., 2003; Eltayeb *et al*., 2007; Prashanth *et al*., 2008).

Superoxide dismutase (SOD) participates in the scavenging of ROS by converting toxic $O^{\cdot-}_2$ (superoxide ions) into H_2O_2 (hydrogen peroxide), which is further detoxified either by the enzymes APX, DHAR and GR through Asada-Halliwell pathway (Salin, 1991; Asada, 1994), or by CAT (Anderson *et al*., 1995; Comba *et al*., 1998) (Fig. 5a). Enzymes in this pathway play very important roles during salt stress (de Azevedo Neto *et al*., 2006). The higher activities of CAT, SOD, and DHAR in Bur oak (Fig. 5b) suggest that this species might have a more efficient

antioxidant system able to cope with salt stress. Comparative studies between salt sensitive and tolerant genotypes revealed higher activities of these enzymes in the latter (Abogadallah, 2010), and experimental manipulations of the same enzymes altered grown behavior under stress conditions. Enhanced stress tolerance was observed in rice plants over-expressing a mangrove SOD (Prashanth *et al*., 2008) and in transgenic tobacco plants with elevated levels of DHAR (Eltayeb *et al*., 2007). While the function of SOD is to scavenge superoxide anions, that of DHAR is to increase the ascorbate (AP) pool through the reduction of dehydroascorbate (Fig. 5a). The contribution of this enzyme in elevating APX levels is crucial in many stress responses, as APX is a key antioxidant enzyme in plants (Foyer and Shigeoka, 2011). Under saline conditions the high activity of DHAR observed in Bur oak might also be sustained by the activity of GR which increased in the presence of NaCl (Fig. 5b). Under stress conditions the two enzymes operate in concert as the product of GR is GSH, the substrate of DHAR (Foyer and Shigeoka, 2011) (Fig. 5a). It is worth noting that the activity of APX is higher in Red oak leaves exposed to 25 mM and 75 mM NaCl. This enzyme is often utilized as a marker of salinity stress and pea plants subjected to 100 mM NaCl showed an increased APX activity, due to a spike in H_2O_2 level (Hernandez *et al*., 1999). Compared to Red oak, salinity induces the expression of GR in Bur oak. The expression of this enzyme is often correlated to salt management, as its activity increases in many tolerant species when exposed to high salt levels (Gueta-Dahan *et al*., 1997; Comba *et al*., 1998; Hernandez *et al*., 2000).

The different enzymatic behavior observed in the two species suggests the existence of diverse detoxification mechanisms operating in Bur and Red oak. SOD, CAT, DHAR, and GR seem to be preferential detoxification enzymes in Bur oak, while APX shows higher activities in Red oak. Enzymatic diversification as a result of salinity stress is not uncommon (Gupta and

Huang, 2014). One of the major functions of an active antioxidant system in leaves is to protect photosynthetic tissue from ROS damage. It is in fact well established that elevated accumulation of superoxide and/or hydrogen peroxide in chloroplasts following abiotic stress conditions compromises many components of the photosynthetic machinery (Foyer and Shigeoka, 2011).

Besides alleviating oxidative damage by limiting ROS accumulation through the activation of the antioxidant system, plants exposed to saline conditions often develop structural barriers in the roots to limit the influx of salt ions (Poljakoff-Mayber, 1975; Grigore *et al*., 2014). The sustained root growth observed in Bur oak exposed to high salt concentration might be at least in part ascribed to structural modifications of the root system limiting ion movement. In this species, maturation of endodermal and peridermal layers, reinforced with Casparian bands and suberin, are noticeable in control (0 mM NaCl) roots at 5 and 10 cm, respectively, from the root tip (Fig. 7a). The presence and strengthening of these physical barriers increase the selectivity of ions able to access the internal layers of the root by limiting apoplastic movement while favoring the symplastic transport regulated by plasmodesmata and specific transporters (Enstone *et al*., 2003; Ma and Peterson, 2003). Development of Casparian bands and suberization close to the root tip has been reported in maize roots experiencing severe salinity stress (Karahara *et al*., 2004), in halophytes (salt-tolerant plants) (Kronzucker and Britto, 2011), and during metal toxicity responses (Lux *et al*., 2004). Compared to Bur oak, the development of Casparian bands and suberization of endodermis and peridermis in Red oak occurs at a greater distance from the root tip (Fig. 7a and 7b). In Red oak, the staining intensity of both berberine hemisulphate and Sudan red is much fainter. These morphological characteristics suggest that Red oak has a reduced ability to restrict the apoplastic movement of toxic ions. It is worth mentioning that these structural differences between Bur and Red oak roots are visible under “un-stressed” (0

mM NaCl) conditions, although they become more apparent upon exposure to NaCl. In both species, development of Casparian bands and suberization can be induced closer to the root tip by salt, but in Bur oak these effects are achievable with lower NaCl levels (Fig. 7c and 7d). It is therefore suggested that besides a natural predisposition, Bur oak is more responsive to modifications of root structure in response to saline conditions.

Exposure to salinity and/or structural modifications of the root system affects the uptake of nutrients and their allocation among organs. Significant differences in macro- and micro-nutrient accumulation were observed in Bur and Red oak (Fig. 6). Relative to Red oak, Bur oak leaves tend to accumulate more Mg^{2+} and Ca^{2+} (Fig. 6a). Maintenance of Mg^{2+} homeostasis in the photosynthetic tissue is crucial for plant survival. Besides being a central element for the proper function of many enzymes, Mg^{2+} is essential for modulating ionic current across membranes which regulates stomatal behavior, and it is a central component of the chlorophyll molecule (Shaul, 2002). Mg^{2+} deficiency has been associated to a decline in photosynthetic rate (Cakmak and Kirkby, 2008). Therefore, retention of Mg^{2+} level in the leaf tissue of Bur oak might explain, at least in part, the better performance of Bur oak plants following exposure to salt. Besides Mg^{2+}, the higher levels of Ca^{2+} in leave tissue of Bur oak might also contribute to the better performance. The role of this element in modulating phosphatase enzymes activity in the carbon reduction cycle, regulating the activity of chloroplast NAD^{+} kinase, and ensuring proper functioning of the reaction centers in the two photosystems has long been recognized (Brand and Becker, 1984).

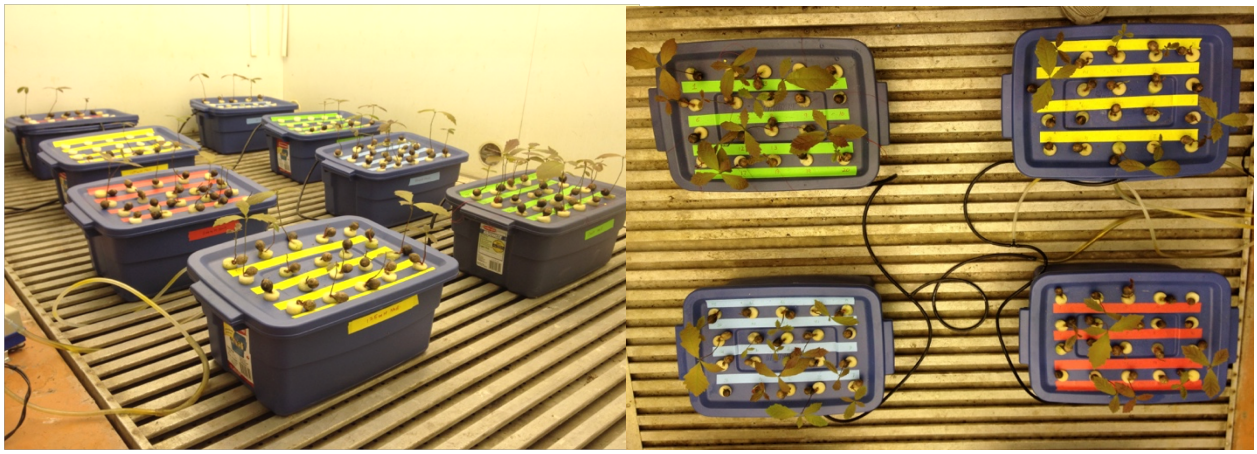
In both oak species, salinity increased the level of leaf Cu with a sharper increment occurring in Bur oak, where Cu level increased by more than 300% from less than 2 ppm at 0 mM NaCl to about 6 ppm at 75 mM NaCl (Fig. 6a). While studies of Cu levels on plant growth

have often been related to the toxicity effects of this element, evidence suggests that Cu might protect the cells from salinity-induced damage. Among its many functions, Cu favors the accumulation of phenolic compounds, which mitigate lipid peroxidation and retain membrane permeability under severe stress conditions, including NaCl exposure (Hejazi *et al*., 2012). Zinc (Zn) levels in leaf and root tissue of Red oak decreased at high salt concentrations whereas remained unaffected in Bur oak (Fig. 6). Besides decreasing the concentrations of soluble proteins and chlorophyll causing a severe reduction in growth, Zn deficiency has an inhibitory effect on SOD and GR (Cakmak and Marshner, 1993), the activity of which decreases in Red oak with elevated levels of NaCl.

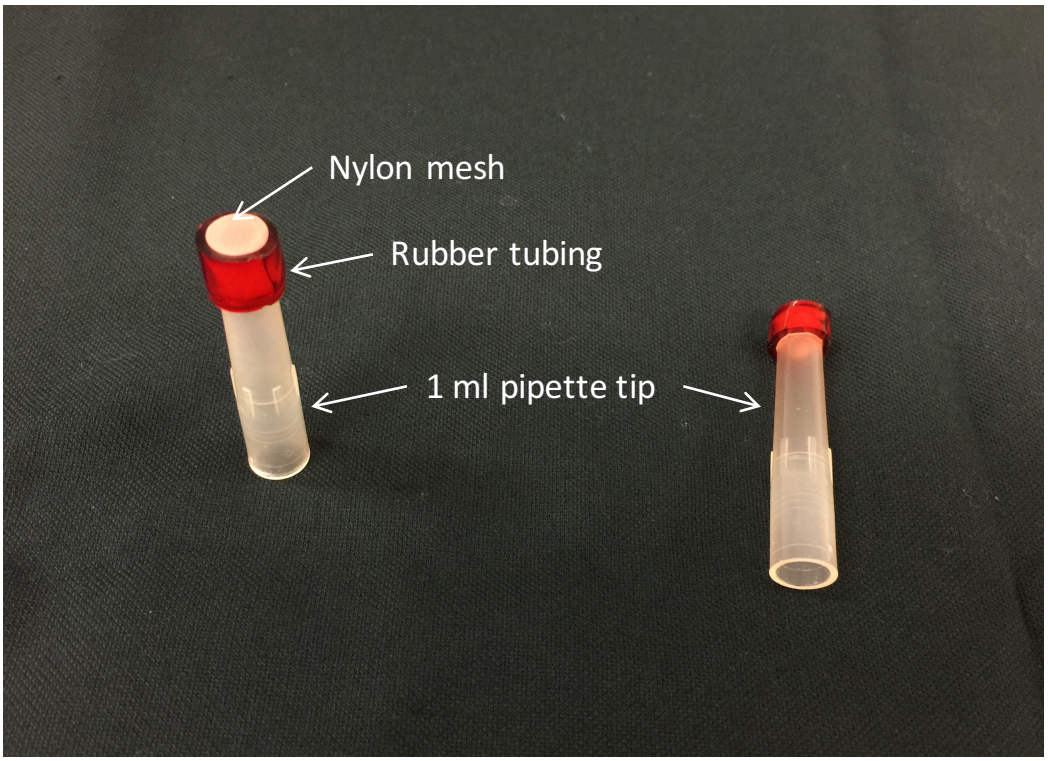
Changes in the amount of two essential macronutrients, nitrogen (N) and potassium (K), were also observed in root and leaf tissues of the two species. The level of N does not seem to be affected greatly, while that of K increased in leaves of Red oak and declined in root tissue of both species. Previous studies revealed that K and N uptake are limited under high salt concentration (Botella *et al*., 1997; Gabr, 1999; Gupta and Huang, 2014).

In conclusion, these studies demonstrate that increasing levels of NaCl restrict root growth, alter nutrient uptake, and induce leaf injury, in both Bur and Red oak. However, the repressive effect of salinity is less pronounced in Bur oak possibly due to morphological characteristics, which include the formation of apoplastic barriers in the root system, leading to increased ion selectivity, as well as physiological adjustments enhancing the antioxidant response.

3.5 Supplement figures



Supplement fig. 1 Hydroponic system with irrigation tubes and air pumps to expose Bur and Red oak seedlings to four different levels of NaCl. Green = 0 mM NaCl (control); Blue = 25 mM NaCl; yellow = 50 mM NaCl; red = 75 mM NaCl.



Supplement fig. 2 Root section holders (constructed using 1 ml pipette tips, nylon mesh and rubber tubing) used to facilitate staining of root sections without tissue deterioration.

4. Chapter 2: Propagation of Bur Oak through cuttings and use of root pruning trays to improve root architecture

4.1 Introduction

In the absence of well-developed propagation systems, Bur oak trees are mainly propagated by seeds. As a cross pollinated species, Bur oak trees grown from seeds are highly heterozygous (Dow and Ashley, 1998; Craft *et al*., 2002; Dutech *et al*., 2005). Even when grown from a single seed source, high variability in desirable traits such as cold hardiness, drought and disease resistance is observed (Dow and Ashley, 1998).

Vegetative propagation allows for the production of identical plants (clones) and the retention of traits in heterozygous species (Acquaah, 2005; Brown, 2008). Using vegetative propagation techniques, it is possible to establish superior populations of selected Bur oak trees with high vigour and improved resistance to biotic and abiotic stress. Besides producing homogenous populations, vegetative propagation has the advantage of shortening the time required to obtain mature plants (Hartmann *et al*., 1997). Vegetative propagation in Bur oak is mainly achieved by grafting, a labor-intensive and time consuming technique (Sumrah *et al.*, 2002; Sutanto, 2010). Therefore, the implementation of new propagation techniques would be extremely beneficial.

Propagation via softwood cuttings is one of the most common methods used in nurseries. It is significantly faster than grafting in that it can produce a large number of ready-to-transplant plants in less than one year (Hartmann *et al*., 1997). Under specific conditions, propagation by cuttings can use any plant material, such as leaves, shoots or roots to regenerate a whole plant (Hartmann *et al*., 1997; Acquaah, 2005). Stems, however, are the preferred cuttings in woody plants (Dirr and Heuser, 1987).

The success of propagation via cuttings depends on several parameters including the age of the stock plant, the time of collection, endogenous hormonal levels, and environmental factors (Burd and Dirr, 1977; Chapman and Hoover, 1981; Tousignant *et al*., 2003; Acquaah, 2005; Rosier *et al*., 2005). Independent studies have reported an increased concentration of root inhibitory compounds, resulting in reduced viability of cuttings, with increased age of the donor plant (Paton *et al*., 1970). Besides natural factors, rooting of softwood cuttings can be enhanced by practices which include the exogenous applications of hormones (Alegre *et al*., 1998) and the utilization of mist systems (Sutanto, 2010). Among hormones, auxins enhance the formation of adventitious roots in cuttings of woody species (Acquaah, 2005). Among the several auxins available, indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are routinely used (Sutanto, 2010), although IBA is often preferred due to its reduced toxicity and mobility in tissue and soil (Al-Salem and Karam, 2001; Kesari *et al.*, 2009). Some studies also showed that IBA in combination with NAA is very effective in promoting root formation from stem cuttings (Basak *et al.,* 2000). Hormones can be applied to cuttings either as solutions or in powder or gel forms (Brown, 2008).

Besides the lack of effective vegetative propagation techniques, the introduction of Bur oak trees in urban ecosystem is hindered by the low transplantation success (Allen and Kuta, 1994) due to the single taproot system (Weaver and Kramer, 1932). These characteristic are in part responsible for the exclusion of Bur oak in urban areas (Allen and Kuta, 1994). Other species like green ash (*Fraxinus* spp.) and elm (*Ulmus* spp.) trees with better transplantation abilities are generally preferred.

Establishment of a vigorous root system plays a more important role than the establishment of the shoot in the frequency of plant survival after transplantation (Hoffmann,

1966; Lyr and Hoffmann, 1967; Harris *et al*., 1995). Unlike shoots, roots continue to grow in autumn, spreading well beyond the shoot system and during transplantation a significant fraction of the root might be damaged and/or not harvested (Watson and Himelick, 1982a; Watson and Sydnor, 1987; Gilman, 1988). The low survival rate of transplanted Bur oak trees is mainly attributed to physical root damage during transplantation (Row *et al*., 2012).

Ideally, for successful transplantation to occur, a plant should have a root ball with a large number of roots to maximize absorption of water and nutrients during the establishment period (Solfjeld and Hansen, 2004). Various root pruning methods are used to shape the architecture of the root system by decreasing the root volume (to eliminate damage during transplanting) while favoring branching. Root pruning is often used to encourage secondary root emergence (Watson and Himelick, 1982a; Watson and Himelick, 1983) and through the multiplication of secondary roots healthier root balls are established (Watson, 1986). Another advantage of root pruning is the ability to reduce growth of the above-ground organs thereby widening the window or time for successful transplantation (Gilman, 1987). In this regard a negative correlation exists between a high transpiration rate (associated with an extensive shoot mass) and the success of transplantation (Broschat and Donselman, 1986). This observation reinforces the idea that a reduction in water loss at the onset of transplantation is crucial for plant survival. Besides limiting transpiration by decreasing shoot mass, root pruning also decreases water loss through a reduction of stomatal conductance (Stupendick and Shephered, 1980; Arnold, 1987; Arnold and Struve, 1989a). Due to these advantages, it is therefore not surprising that many different types of root pruning containers have been developed (Arnold and Struve, 1989b).

RootMaker® root pruning containers are one of the most efficient air pruning systems available for extensive and efficient pruning of plant roots in a variety of species. Plants grown in these containers exhibit fibrous and non-girdling root balls, enhanced water retention and nutrient absorption, as well as greater survival following transplantation (Gilman and Yeager, 1987; Marler and Willis, 1996). However, to my knowledge, the effect of root pruning containers has not been studied in Bur oak.

The objectives of this study are to 1) assess the effects of exogenous hormonal applications, age of donor plant, and time of cutting collection on rooting in semi-hardwood cuttings of Bur oak, and 2) conduct preliminary work on the effects of root pruning on Bur and Red oak genotypes using RootMaker® trays.

4.2 Materials and methods

4.2.1 Rooting of semi-hardwood cuttings

This experiment was conducted in two consecutive years. Based on the results obtained from the first year (2012) some modifications were made in the experimental setup in the second year (2013).

4.2.1.1 Experimental year 2012

Plant materials and date of collection

During 2012, semi-hardwood cuttings were collected from Bur oak plants of three different age groups, mature trees (10-15 years old), juvenile trees (3-4 years old) and seedlings (1 year old), to test the effect of age of the plant on rooting (Rosier *et al*., 2005). These trees were located at the Young Farm of Jeffries Nurseries Ltd. and at Riverbend Orchards Inc., both in Portage La

Prairie, Manitoba. The cuttings were collected on July 24 and August 2 and stored in cold water until planting. The cuttings were 10-15 cm in length and only 2-3 leaves were retained in order to prevent transpiration losses while maintaining photosynthetic efficiency.

Hormone applications

To enhance rooting, cuttings were treated with different concentrations and combinations of indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) (Table 2). The two hormone solutions were prepared by dissolving the appropriate amount of IBA (Sigma-Aldrich® I5386) and NAA (Sigma-Aldrich® N-0640) in 1 M sodium hydroxide (NaOH). The solutions were stored in the dark at 4°C. The basal (2-3 cm) end of the cuttings was slightly wounded with a serrated knife, dipped in the hormone solutions for 30 seconds, and planted in Ellepot trays. Two biological replicates consisting of 9 plants each were used per collection day and treatment. Control cuttings were treated with 1M NaOH.

Rooting conditions

Cuttings were rooted in a greenhouse at Riverbend Orchards Inc. (Fig. 8). The greenhouse was equipped with an intermittent misting system and automated exhaust fan to control temperature and moisture levels, key conditions for successful plant regeneration (Sutanto, 2010). After 4 weeks the rooting system of the cuttings was analysed using a numerical scale (0-5) estimating callus growth and root development (Fig. 9).

Table 2 Different combination of IBA and NAA used to stimulate rooting in semi-hardwood cuttings of Bur oak (*Q. macrocarpa*) collected in 2012 and 2013. Hormones were dissolved in 1 M sodium hydroxide (NaOH).

Rooting hormone	**Concentration (%) (Year 2012)**	**Concentration (%) (Year 2013)**
Indole-3-butyric acid (IBA) +1-Naphthaleneacetic acid (NAA)	0.1+0.1	0.1+0.1
	0.5+0.25	0.5+0.25
	0.5+0.5	
	1+0.5	
Indole-3-butyric acid (IBA)	0.05	0.05
	0.1	0.1
	0.5	0.5
	1	

Fig. 8 Plastic covered greenhouse with an overhead misting system at Riverbend Orchards Inc., Portage la Prairie, Manitoba. Bur oak cuttings were grown in Ellepot trays for 4 weeks (picture taken: 4 weeks after planting).

4.2.1.2 Experimental year 2013

Based on the results obtained from the 2012 cutting experiments, only 5 hormonal treatments were used in 2013 (Table 2). Furthermore, cuttings were collected only in August due to the long winter experienced in the previous year. Time of hormone exposure was also tested in 2013 by applying hormones for 30 and 120 seconds.

Collection of cuttings and growth conditions were similar to those described for 2012.

Scale	Extend of rooting
0	no callus or root formation
1	little callus formation
2	moderate callus formation
3	high amount of callus formation
4	full root covered with callus
5	presence of roots

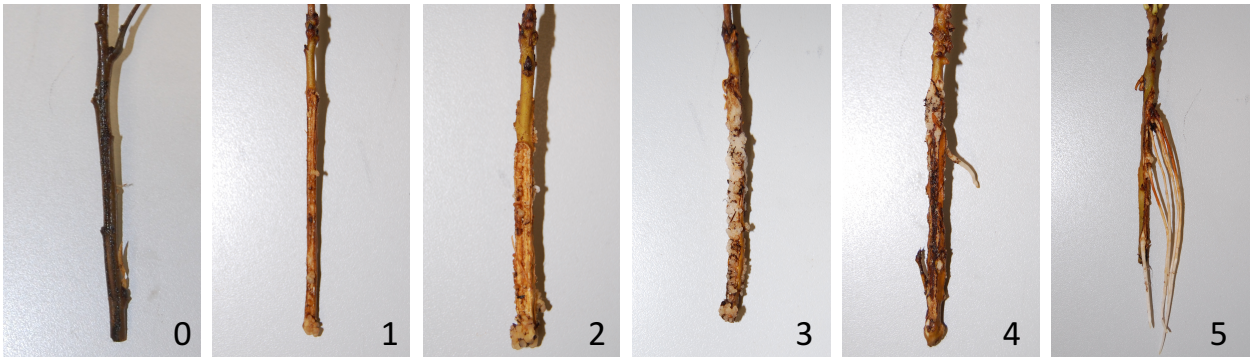


Fig. 9 Numerical scale (0-5) used to analyze the extend of rooting in Bur oak (*Q. macrocarpa*) cuttings after 4 weeks of growth.

4.2.2 Effect of rooting containers on root structure

Acorns from 4 different Bur oak and 1 Red oak genotypes were planted in sand:soil:peat (2:1:1, V/V) using three different types of containers: RootMaker® trays (for pruning at multiple points), Ellepot trays (for pruning at one point) (Fig.10), and large pots (to mimic field conditions). After stratification at 4°C for 3 months to achieve uniform germination, the acorns were transferred to the greenhouse at the University of Manitoba, Canada, (22 ± 5°C and 16h daylight), watered on alternate days, and fertilized with 20:20:20:: N:P:K every week. After 4 months, roots were analysed.

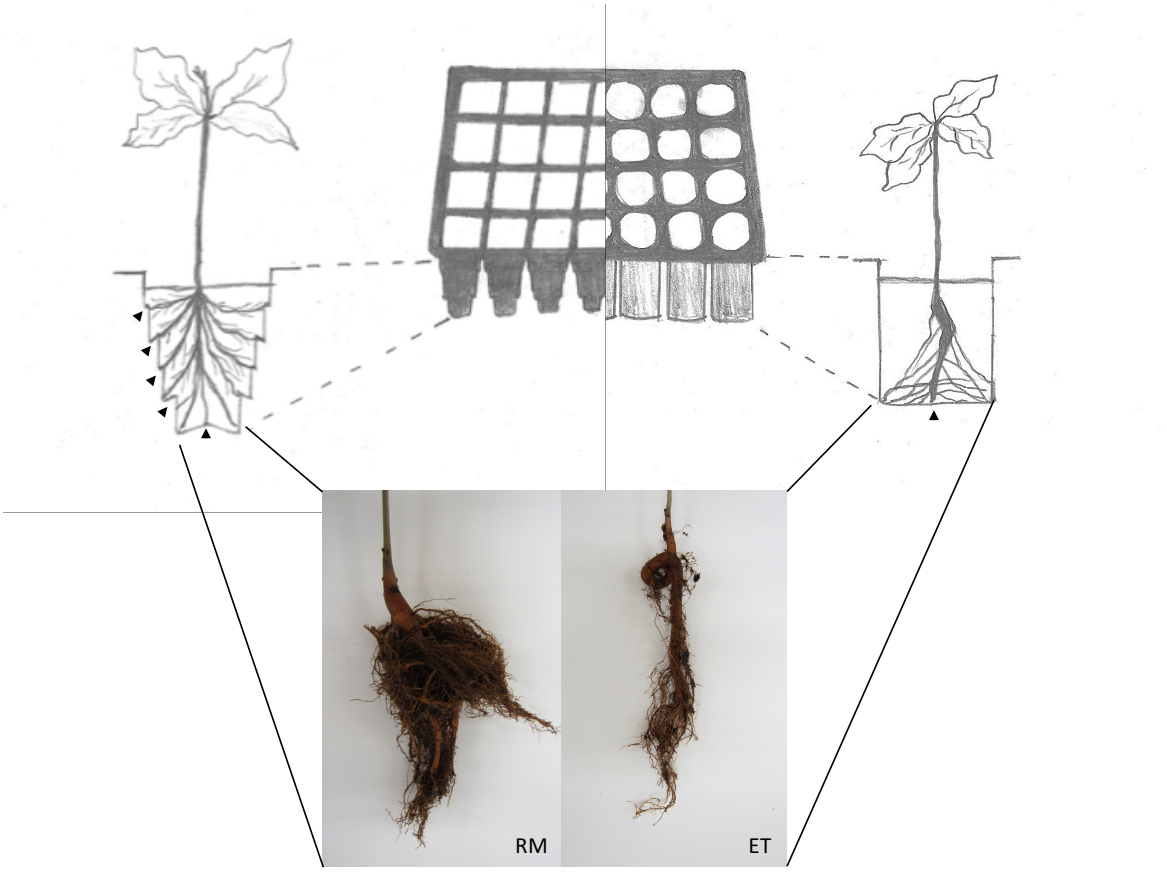


Fig. 10 Effect of root pruning with RootMaker® trays (left) and Ellepot trays (right) on root structure of Bur oak seedlings. Pictures show roots structures of seedlings after 16 weeks of growth in RootMaker® trays (RM) and Ellepot trays (ET); Arrowheads = points of root pruning

4.2.3 Statistical analysis

Experiments were designed using a completely randomized design to limit experimental errors and to improve efficiency of statistical testing. Data were analyzed by two-way ANOVA in SPSS program (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. IBM Corp., Armonk, NY, USA). Differences between various parameters were tested by comparing treatments means from ANOVA test using Duncan's multiple range test (a = 0.05).

4.3 Results

4.3.1 Rooting of semi-hardwood cuttings

4.3.1.1 Experimental year 2012

This experiment was designed to examine the influence of time of collection, age of donor plant, and concentration of hormones on regeneration of hardwood Bur oak cuttings. The experimental design included semi-hardwood cuttings from three age groups of plants collected at two different times of the year and nine hormonal treatments. After 4 weeks of growth only cuttings collected from 1 year old seedlings retained some green leaves, while those collected from juvenile (10-15 years old) trees exhibited senescence (Fig. 11). Older cuttings (from 10-15 years old trees) died after the four weeks.

In cuttings generated from 1 year old seedlings, root formation was encouraged by different hormonal combinations: 0.1% IBA, 0.5% IBA, 0.05% IBA, 0.1% IBA+0.1% NAA and 0.5% IBA+0.25% NAA (Fig. 12a and 12b). A less pronounced inductive effect was observed in cuttings produced by juvenile (10-15 years old) trees when treated with 0.1% IBA+0.1% NAA, 0.1% IBA and 0.5% IBA (Fig. 12c). No formation of callus or root was ever observed in cuttings collected from old trees.

Fig. 11 Bur oak (*Q. macrocarpa*) cuttings collected from 1 year old seedlings (left) or from juvenile (3-4 years old trees) (right) after 4 weeks of growth.

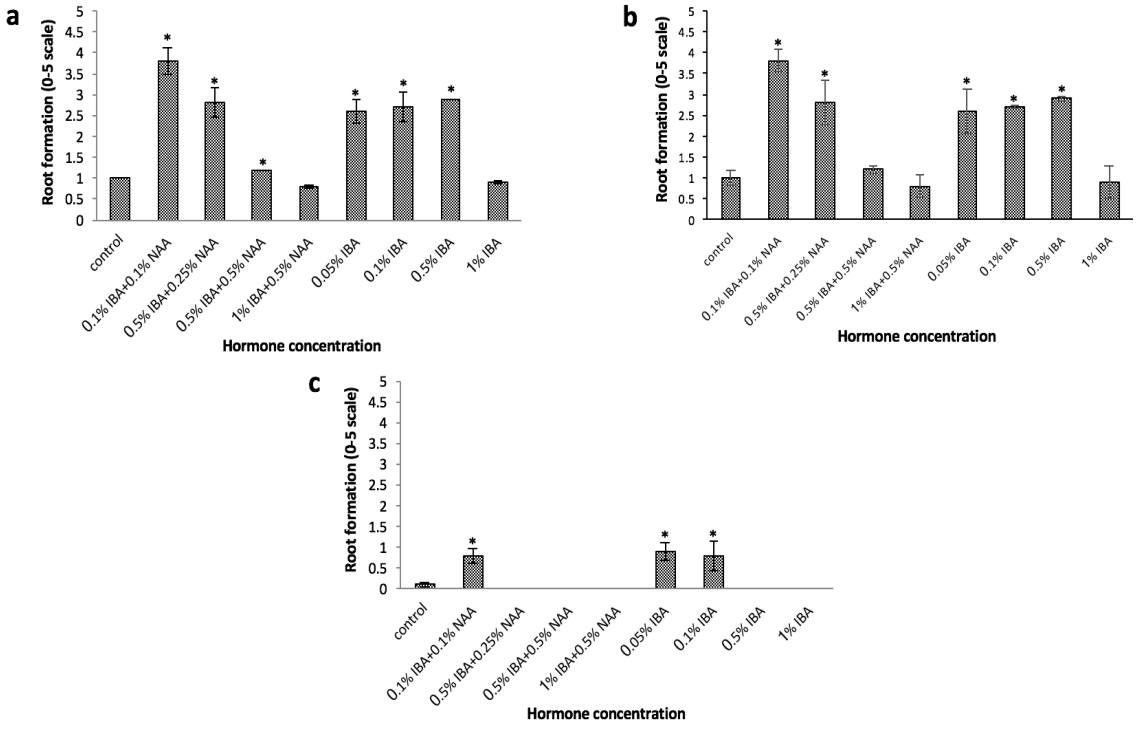


Fig. 12 Effect of different levels of hormones on rooting of semi-hardwood cuttings of Bur oak collected from 1 year old seedlings on July 23, 2012 **(a)** or August 2, 2012 **(b),** or from juvenile (3-4 years old) plants collected on August 2, 2012 **(c)**. Values ± SE are means of three biological replicates. Asterisks indicate significantly higher values ($p < 0.05$) relative to control.

4.3.1.2 Experimental year 2013

During the 2013 experimental season, the five hormonal treatments inducing rooting in 2012 (Table 2 and Fig. 12) were used for 30 secs and 120 secs. Unfortunately, all the cuttings died prematurely with no signs of callus and/or roots. This was probably due to the extremely cold winter experienced that year, which allowed only a small window of time for collection (first week in August).

4.3.2 Effect of rooting containers on root structure

The effect of rooting containers (RootMaker® trays, Ellepot trays, and large pots) on root morphology was examined in both 2012 and 2013 seasons with similar results. After 16 weeks of growth, cuttings in large pots exhibited an elongated root system with thin secondary roots emerging from a dominant primary root. This was in contrast to cuttings grown in RootMaker® trays and Ellepot trays, which had low-volume root balls characterized by many lateral roots (Fig. 13). A higher number of highly branched fine roots was observed in plants grown in RootMaker® trays compared to Ellepot trays These phenotypes were consistent among all genotypes of Bur and Red oak analysed.

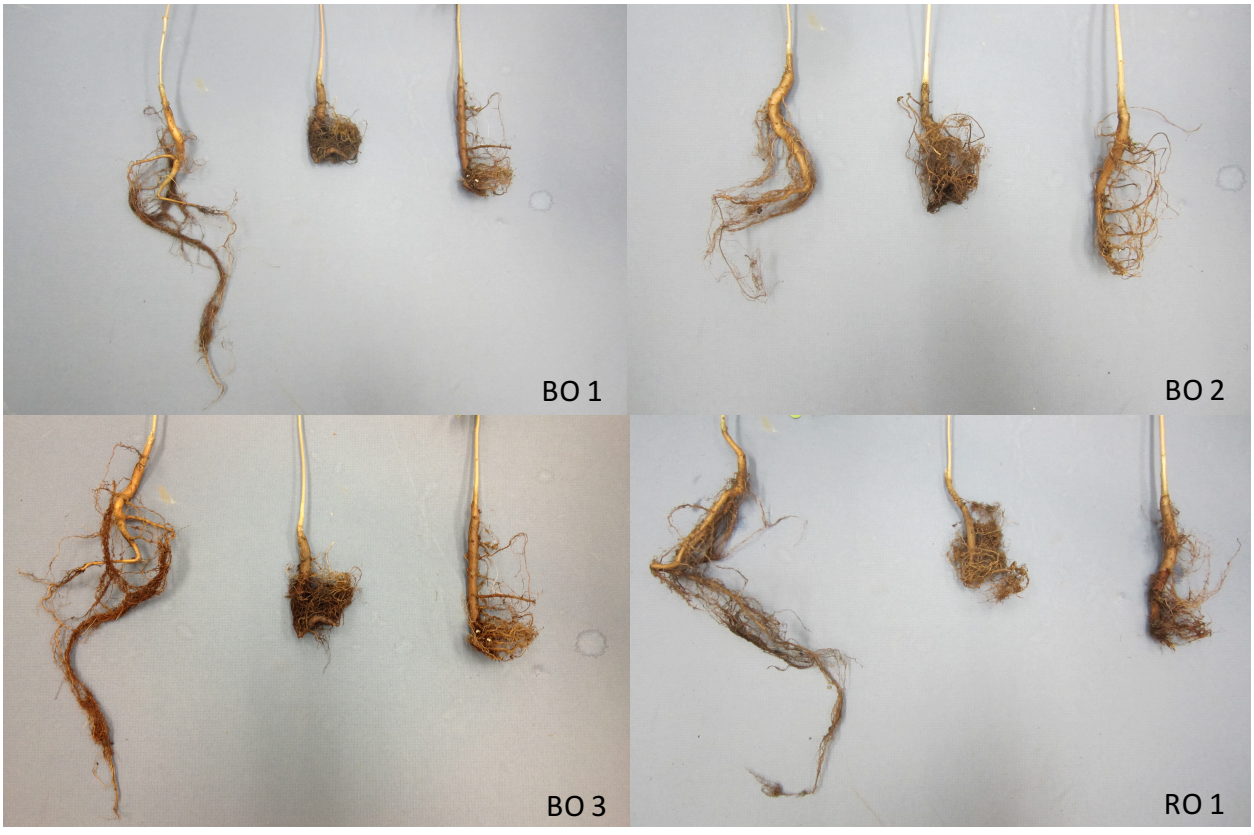


Fig. 13 Root structure of three genotypes of Bur oak (BO1-3) and one of Red oak (RO1) seedlings after 16 weeks of growth in large pots (left), RootMaker® trays (centre), and Ellepot trays (right).

4.4 Discussion

4.4.1 Semi-hardwood cuttings

The age of donor plant plays a significant role in determining the success of rooting in cuttings (Greenwood and Hutchison, 1993), an observation also true for Bur oak. Cuttings collected from 1 year old seedlings possessed significantly higher rooting ability compared to those generated from juvenile (3-4 years old) trees (Fig. 12), while no rooting was observed in cuttings obtained from mature (10-15 years old) trees. These results are consistent with those of Richer *et al*. (2003) who described a negative relationship between age of *A. saccharum* trees and rooting potential. Bhusal *et al.* (2003) also reported better rooting in cuttings collected from juvenile plants as compared to adult stock plants. Dirr and Heuser (1987) suggested that the ability to produce successful cuttings from old plants is greatly reduced by the accumulation of as yet unknown dormancy-inducing compounds.

Besides age, environmental factors are also important for the successful establishment of cuttings (Richer *et al*., 2003). Better environmental conditions of the donor plant might indeed favor the performance of cuttings, an observation consistent with the current results. Cuttings exhibiting better rooting ability were those obtained from 1 year old seedlings grown in the greenhouse. Leaf size and morphological characteristics might also have affected the ability to form roots. Relative to cuttings obtained from juvenile and mature trees, which were characterized by larger leaves, those generated from 1 year old seedlings had smaller leaves, which might have prevented excessive water loss due to transpiration after transplanting. Smaller leaves also have a thin epidermis and reduced cuticle (Dudley, 1996), features allowing better water penetration from the misting systems. The importance of healthy and functional leaves during cuttings has been examined by Middleton *et al*. (1980) who reported that external

hormonal applications on cuttings encourage rooting only when leaves are present. Leaves play a critical role during the establishment of cuttings by providing essential sugars to sustain growth until roots are formed (Nanda and Anand, 1970).

Application of exogenous hormones is another major factor influencing root growth in cuttings. External application of auxins has been proven to successfully promote root emergence in stem cuttings in a number of species (Fogaca and Fett-Neto, 2005; Kesari *et al.*, 2009; Vakouftsis *et al*., 2009). Besides the nature of the applied hormone, cuttings are very sensitive to different hormonal concentrations (Al-Salem and Karam, 2001; Kesari *et al.*, 2009). In Bur oak both IBA and IBA+NAA were successful in inducing rooting at low concentrations, with higher concentrations having an inhibitory effect (Fig. 12). These results are supported by another report where among five different IBA concentrations, the lowest concentration (20 µg/cutting) produced the larger number of roots from stem cuttings of *S. leprosula* (Aminah et al., 1995). In the same experiment it was shown that root density decreased significantly as the hormone levels increased. In agreement with this observation, stem cuttings of *Milicia excelsa* were only able to generate roots at IBA concentrations lower than 0.2%, whereas no roots were produced in treatments with more than 0.2% IBA (Ofori, 1996). In the case of *P. pinnata* although all concentrations of auxin were able to start root sprouting from cuttings, complete formation of roots was only observed at the lowest concentrations (Kesari *et al.*, 2009).

Optimal combination of hormones is also paramount for successful rooting. In Bur oak, among all hormone combinations used, 0.1% IBA+0.1% NAA was the most successful in all cutting types (Fig. 12). Similar results were also reported in *Cynometra iripa*, *Excoecaria agallocha* and *Heritiera fomes* trees where stem cuttings subjected to IBA+NAA solution

produced more roots than any other combinations of indole-3-acetic acid (IAA), IBA and NAA (Basak *et al.*, 2000).

Rooting experiments in Bur oak were conducted during the 2012 and 2013 seasons. The results presented in this thesis only refer to the 2012 season, as no root or callus formation was observed in 2013, irrespective of the hormonal treatments. Failure to produce roots during this season was ascribed to the harsh winter that kept plants dormant for a longer period of time in spring. The efficiency of exogenous auxin during rooting is influenced by seasonal changes (Nanda *et al.*, 1968). Furthermore, fluctuations in environmental conditions and severe weather are known to affect many physiological events, including the activity of several hydrolyzing enzymes required for the mobilization of starch from the photosynthetic tissue to the emerging roots (Nanda and Anand, 1970). All these events might have contributed to the lack of rooting observed in 2013.

4.4.2 Effect of rooting trays on root structure

Pruning in RootMaker® trays occurred throughout the entire length of root resulting in a high number of lateral roots. This was in contrast to Ellepot trays were pruning was restricted to a small section of the root, and plants exhibited a smaller number of lateral roots (Fig. 10 and Fig. 13). In the absence of pruning, as in the case of plants grown in larger pots, plants develop a long primary with very few lateral roots. These findings emphasize the importance of root pruning to achieve better root structure in oak with healthier and highly branched roots. The results obtained in this study agree with those of Solfjeld and Hansen (2004) and Arnold and Young (1991) showing enhanced root formation in Sabal palm and apple (*M. domestica*) following pruning. In the present experiment, the effects of the tray type on root architecture were similar among

species (Bur and Red oak) and genotypes; an observation inconsistent with the studies of Solfjeld and Hansen (2004) and Wilson *et al*. (1997) who documented different responses among genotypes.

The characteristic root balls obtained with RootMaker® trays (Fig. 13) increase the chances of survival following transplanting as they provide a larger surface area for water and nutrient uptake. Furthermore, the larger number of lateral roots greatly increase the performance of the plant to new microenvironments, as revealed in southern live oak (*Q. virginiana* L.) (Gilman and Yeager, 1987), sweetgum (Kormanik, 1986), and northern Red oak (Ruehle and Kormanik, 1986).

Collectively, results from this experiment emphasize the importance of the age of the donor plant and hormonal applications on the vegetative propagation of Bur oak. Use of RootMaker® trays can produce healthier root ball and higher root branching in both Bur oak and Red oak trees. These improvements in root structure can singnificatly increase the potential of these trees to be successfully transplanted from nurseries to urban settings. While still very preliminary, these results can be used as an initial experimental framework that can be further optimized to propagate oak species with enhanced characteristics.

5. General discussion and conclusions

The response of Bur and Red oak to salinity was examined in Chapter 1. Relative to Red oak, Bur oak seedlings exposed to elevated levels of NaCl exhibited enhanced root growth and reduced leaf damage, denoting a better tolerance to salt stress. Development of salt tolerance was examined at anatomical and physiological levels. High concentrations of NaCl induced the formation of casparian bands and suberin lamella in the roots of both species; however, in Bur oak these anatomical changes occurred closer to the root tip in lower salt levels. Development of casparian bands and suberin lamellas in proximity of the root meristem were reported to enhance salt tolerance in red-osier dogwood (Davis *et al*., 2014) as they limit the apoplastic movement, thereby allowing the plant to control the intake of toxic ions. Furthermore, both casparian bands and suberin lamella were also visible in Bur oak roots grown in the absence of NaCl, thus suggesting a "natural" predisposition of this species towards the symplastic movement of nutrients. This characteristic, observed in several salt-tolerant plants (halophytes) (Grigore *et al.*, 2014), furthers reinforces the idea that Bur oak is more tolerant to saline conditions than Red oak.

Salinity like many other forms of stress elevates the levels of ROS by increasing their biosynthesis and/or reducing the ROS scavenging mechanisms mediated by the cellular antioxidant enzymes (Foyer and Noctor, 2003; Tuteja, 2007). Compared to Red oak, the activities of several antioxidant enzymes, such as SOD, CAT, POD, DHAR and GR are higher in Bur oak leaves in the presence of high salt levels. These enzymes have been shown to reduce the amount of ROS, thereby protecting cellular components and enhancing the performance of plants grown under sub-optimal environmental conditions, including salinity (Rodriguez-Rosales *et al*., 1999; Rios-Gonzalez *et al*., 2002; Abogadallah, 2010). Retention of photosynthetic rate and

execution of basic metabolic processes were observed in plants grown in NaCl following an experimental increase in the activity of one or more of the antioxidant enzymes (Mittova *et al*., 2003; Eltayeb *et al*., 2007; Prashanth *et al*., 2008).

Understanding the response of Bur and Red oak seedlings to salt stress is valuable in assessing the potential of introducing these species in urban areas characterized by high salinity as a result of excessive use of de-icing salt on roadways. From these studies it emerged that Bur oak might be a better candidate than Red oak. It must be mentioned, however, that studies in this thesis only examine the effect of a short salt exposure, without assessing the effects of long term exposures which might result in different physiological adjustments/responses. Moreover, Bur oak trees are introduced to saline urban soils at older age, after their transplantation from nursery fields into urban settings. Therefore, before making final recommendations, effects of salt stress should also be studied on older Bur oak trees. Salt spray from moving vehicles has also been known to cause significant damage to new growth during spring season (Viskari and Kärenlampi, 2000 Ramakrishna and Viraraghavan, 2005; Hanslin, 2011). Therefore, effects of aerial salt uptake and its deposition of foliage are also needed to be examined.

The second chapter examines the potential of propagating Bur and Red oak via cuttings by assessing the effects of several parameters including age of the donor plant, date of collection and exogenous hormonal applications. While the majority of the experiments were only conducted in one experimental season due to the harsh winter experienced in 2013, it is apparent that successful regeneration via cuttings is correlated to the age of the donor plant, with cuttings from younger plants performing better than older ones. The reason for this observation is most likely due to the different physiological conditions of the cuttings and the presence of possible "inhibitory" substances in cuttings harvested from older trees, which compromised their rooting

ability. It is also apparent that rooting is affected by exogenous hormone applications with best results observed with a combination of IBA and NAA. Low levels of both hormones produced the best response, and further studies on more combinations are recommended to further improve rooting. Given the interaction between hormone and sugars in plant signaling (Nanda *et al.*, 1971) it might be worth examining the effects of combined applications of hormone and sugars.

Results from this experiment only provide information about the effects of age of donor plant and hormonal concentration on root formation in Bur oak cuttings. Other parameters should be included to better interpret these results. For example, growing degree days (GDDs), the number of days for which plants receive optimum temperature for growth, could be also included in further studies (Tousignant *et al*., 2003; Sutanto, 2010). The use of GDDs can minimize the effects of variable weather conditions on rooting in semi-hardwood cuttings of Bur oak.

Moreover during this experiment, only seedlings and juvenile trees were able to produce rooting in their cuttings. This might not be very valuable for the selection of desirable trees, as selection is generally conducted on older plants. Therefore, propagation procedures for older trees need to be optimized using the preliminary findings presented in this thesis.

Chapter 2 also analyzed the effects of root pruning on root architecture. Compared to Ellepot trays, use of RootMakers® trays improved the root architecture in both Bur and Red oak. Seedlings grown in RootMakers® trays formed bigger root balls and produced a higher number of lateral roots compared to those grown in Ellepots trays. These root characteristics improve survival of plants post-transplantation (Ruehle and Kormanik, 1986).

While still preliminary, the results from this thesis provide the basis for further studies on salinity and means to propagate Bur and Red oak in an effort to accelerate their introduction in urban areas.

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