Effects of Sodium Chloride on *Cornus stolonifera*: Responses of Actively Growing Seedlings and of Seedlings during Bud Break

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Abstract

Cornus stolonifera has been identified as a relatively salt tolerant species compared to other species of the boreal forest. Understanding the effects of NaCl on *C. stolonifera* is necessary as this species has been recommended for the reclamation of saline oil sands tailings. Both actively growing and dormant seedlings may be planted on sites to be reclaimed. The effect of salt stress on both actively growing and dormant seedlings was examined. Seedlings were treated hydroponically with NaCl. Shoot dry weight, stomatal conductance, transpiration and photosynthetic rates were reduced by NaCl in actively growing seedlings. The amount of hemicellulose in the cell wall of actively growing seedlings increased during salt stress. Plant water relations, cell wall elasticity and the carbohydrate composition of the pectin and hemicellulose fractions were not affected by the salt stress. Dormant seedlings treated with NaCl during bud break had lower shoot dry weights and shoot heights than untreated seedlings. The cell walls of seedlings treated with NaCl during bud break were more rigid, but the plant water relations suggest that this change did not contribute to salt tolerance. Salt stress did not alter the cell wall composition of seedlings treated with NaCl during bud break. The results of this study suggest that actively growing *C. stolonifera* seedlings may be more salt tolerant than seedlings during bud break; however, more work will need to be done to gain a more complete understanding of salt tolerance in *C. stolonifera.*

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1.0 General Introduction

Plants experience varying types and levels of stress, which are defined as adverse forces or factors that inhibit normal plant functioning (Jones and Jones 1989). Sodium chloride is particularly detrimental to plants as it causes both osmotic stress and ion toxicity resulting in reduced growth and often death. Osmotic stress results from high concentrations of Na^+ and Cl^- in the soil that decrease the soil water potential, reducing the ability of the plant to absorb water (Cramer 1997; Volkmar et al. 1998). Ion toxicity occurs when high levels of Na^+ and Cl^- accumulate in the plant or move through plant tissue, affecting metabolism, enzyme activity, membrane integrity and nutrient uptake (Greenway and Munns 1980; Cramer 1997; Volkmar et al. 1998).

The number of plants experiencing salt stress is rising as agricultural and industrial activities are causing secondary soil salinization. Oil sands mining is an industrial activity of particular interest as the amount of saline tailings produced is greater than that of any other type of mineral processing (Marshall 1982; Van den Heuvel et al. 1999). Reclamation of oil sands tailings is difficult as they are unstable and contain salts, hydrocarbons, and very little organic matter (Van de Heuvel et al. 1999). Sodium chloride is the major electrolyte present in the tailings and reduces the ability of plants to establish and grow during reclamation. Red-osier dogwood (*Cornus stolonifera*) has been recommended as a good species for the reclamation of oil sands tailings in northeastern Alberta as it is relatively salt tolerant compared with other species of the boreal forest (Renault et al. 2000). *Cornus stolonifera* stabilizes soil well, grows rapidly and establishes relatively easily on degraded sites (Crane 1989).

Previous work with *C. stolonifera* has indicated that it is able to restrict the movement of Na^+ and Cl^- into shoot tissue, reducing ion accumulation and thus ion toxicity (Renault et al. 2001). The presence of mechanisms to reduce ion toxicity in *C. stolonifera* indicates that there must also be mechanisms to reduce the effects of

osmotic stress, as osmotic stress is considered to be the most detrimental short-term effect of salinity (Munns and Termat 1986). The mechanisms of osmotic stress tolerance for *C. stolonifera* are not yet known. Adjustments in cell wall elasticity and the accumulation of compatible osmolytes contribute to osmotic stress tolerance in other plant species (Radin 1983; Fan et al. 1994; Volkmar et al. 1998). An increase in cell wall elasticity allows the plant to maintain turgor at lower volumes of water, as the cell wall is able to shrink around the cell contents (Ruiz-Sanchez et al. 1997). A decrease in cell wall elasticity rapidly reduces the pressure potential, helping to decrease the plant water potential and maintain water uptake (Bowman and Roberts 1985). In order to fully understand the contribution of changes in cell wall elasticity to salt tolerance, plant water relation parameters must also be studied. Sodium chloride stress not only alters cell wall elasticity, but can also affect cell wall composition by changing both cell wall physical (Iraki et al. 1989; Neumann et al. 1994) and structural properties (Solomon et al. 1987; Zhong and Lauchli 1993).

Actively growing and dormant *C. stolonifera* seedlings may be planted during reclamation, and thus it is important to understand the effects of salt stress at various plant developmental stages. Reductions in photoperiod and temperature that naturally occur during the fall increase frost resistance in temperate perennial plants. Exposure to low, but not freezing temperatures, is the first stage in cold acclimation and often induces plants to become dormant lacking visible growth (Lang 1996). The physiological, biochemical and molecular changes that occur during cold acclimation have been well studied (Hughes and Dunn 1996; Thomashow 2001), but the effect of these changes on stress tolerance during bud break has not been. The relative water content and transpiration rates are reduced (McKenzie et al. 1974; Parsons 1978) and the amount of soluble carbohydrates are increased (Ashworth et al. 1993) in *C. stolonifera* seedlings during cold acclimation. Accumulation of a 24-KDa dehydrin protein occurs in *C. stolonifera* during cold acclimation (Sarnighausen et al. 2002). These changes may alter the stress tolerance of *C. stolonifera* seedlings when exposed to NaCl stress during bud break.

The overall objective of this study was to gain a better understanding of the effects of salt stress on *C. stolonifera* and the salt tolerance mechanisms that this species uses to tolerate relatively saline conditions. The focus of this study was primarily on cell wall elasticity and composition. Growth, transpiration, stomatal conductance, photosynthesis, chlorophyll content, shoot osmotic potential at full turgor, shoot osmotic potential at turgor loss, shoot pressure potential at full turgor, shoot relative water content at turgor loss, shoot water potential and the amount of soluble carbohydrates were also determined to gain a more complete understanding of how *C. stolonifera* seedlings are affected by NaCl stress. Two separate experiments were done. The first one examined the effects of NaCl on actively growing seedlings and the second one focused on the effects of salt stress on dormant seedlings during bud break. It was hypothesized that NaCl stress would reduce growth and alter water relation parameters in both actively growing and dormant *C. stolonifera* seedlings. Changes observed in cell wall elasticity and composition were predicted to contribute to increased salt tolerance.

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2.0 Literature Review

2.1 Secondary Salinization

Saline soils occur naturally throughout the world. In these saline ecosystems, such as salt marshes, native plant species tolerate salt stress. However, many other species are not tolerant and salt reduces their growth and often results in plant death. Increasingly, due to agriculture and industrial practices, there is an influx of salt into areas that were not previously saline. This secondary salinization degrades natural habitats, severely impacts native vegetation and makes reclamation extremely difficult. On agricultural land, secondary salinization is not a recent problem. For instance the end of the Mesopotamian Empire was thought to be a result of decreased food production due to the salinization of farm land (Jacobsen and Adam 1958). Not only do increased salt levels impact agricultural land but other environments such as areas of the boreal forest are becoming relatively saline due to industrial activities.

A number of activities and practices contribute to secondary salinization; these include but are not limited to the use of deicing salts on roadways, potash mining and oil sands mining. Deicing salts (NaCl and $CaCl_2$) affect roadside plants through aerial drift (Lumis et al. 1973), saline runoff (Westling 1969), or as spray from passing cars (Bogemans et al. 1989; Shannon et al. 1994). Mining activity brings large volumes of salt to the surface through ore extraction and different salts, such as sodium hydroxide, are often used during processing to improve ore extraction (Kotylar et al. 1996). Oil sands mining is particularly detrimental to the surrounding environment as the land required to dispose of the billions of cubic meters of tailings is greater than that of any other type of mineral processing (Marshall 1982; Van den Heuvel et al. 1999).

Oil sands mining in Canada occurs in northeastern Alberta and accounts for 35% of Canada's daily petroleum production (Almdal 2000). The extraction of bitumen from Canadian oil sands is expected to increase, as the deposits are estimated to contain one third of the world's recoverable oil (Van den Heuvel et al. 1999). As

mining operations continue to grow the volume of saline tailings produced will severely impact the environment in northeastern Alberta if they are not successfully reclaimed.

Surface mined oil sands are mixed with hot water and a small amount of sodium hydroxide to extract the bitumen. This extraction processes is called the 'hot water extraction process' (HWEP) (Kotylar et al. 1996), or the 'hot water flotation method' (Van den Heuvel et al. 1999). HWEP produces large volumes of tailings that are retained in sedimentation ponds. As the tailings settle, they separate into coarse tailings sand, fine tailings and free water that is recycled for subsequent extractions (Kotylar et al. 1996). Fine tailings have poor settling properties and consolidate very slowly. They contain salts, hydrocarbons (naphthenic acids) and very little organic matter (Van de Heuvel et al. 1999). Sodium chloride (NaCl) is the major electrolyte present in the tailings, due primarily to the addition of sodium hydroxide and the release of Na^+ and Cl^- from the ore during processing (Kotylar et al. 1996). As water is recycled during subsequent extractions the Na^+ and Cl^- concentration is increased (Kotylar et al. 1996). The amount of NaCl found in the tailings water depends on the concentration of NaCl in the ore body. Future expansion of the oil sands industry is predicted to encounter ore bodies with increased salt levels (Kotylar et al. 1996).

To increase the rate at which the fine tailings become trafficable and reclaimable the fine tailings are mixed with coarse tailings, and gypsum is added to initiate coagulation (Van den Heuvel et al. 1999; Franklin et al. 2002). Tailings that have been treated in this manner are termed consolidated or composite tailings (CT) (Van den Heuvel et al. 1999). As CT solidifies, CT water is released. Although CT solidify more rapidly than fine tailings, both the CT and CT water contain higher ion concentrations, particularly Na^+ and Cl^-, in the form of NaCl and Na_2SO_4 (Redfield and Zwiazek 2000). The high salinity of both the fine tailings and CT decreases the ability of plant species to successfully establish on tailing sites that are to be reclaimed.

2.2 Effects of Salt Stress

The primary salt that causes high soil salinity and subsequent plant stress is NaCl (Hasegawa et al. 2000). The effect of NaCl on plants varies with species, salt concentration, environmental conditions and plant life history (Greenway and Munns 1980). The salt sensitivity of different species is a continuum and only the extremes are easily classified as salt tolerant halophytes, or salt sensitive glycophytes (Greenway and Munns 1980). Halophytes often have specialized mechanisms such as succulence or structures such as salt glands that help them tolerate high salinity.

Salt stress causes both osmotic and ionic stress. Osmotic stress occurs because high soil salt concentrations decrease the soil water potential (Cramer 1997), making it difficult for water to move across root cell membranes into the plant (Volkmar et al. 1998). Ion toxicity occurs when excess levels of Na^+ and Cl^- accumulate or move through the plant damaging membranes, affecting nutrient uptake, disrupting enzyme activity, and causing cellular dehydration due to apoplastic accumulation of ions (Volkmar et al. 1998).

2.2.1 Osmotic Effects

Although the osmotic effects of salt stress are similar to the effects of water stress, both result in a water deficit for the plant (Munns 2002), the two stresses are different. During drought stress little water is present in the soil, while during salt stress water is usually present but unavailable to the plant due to the decreased soil water potential. Water deficit negatively affects transpiration, metabolite balance, ion movement, nutrient uptake, membrane integrity, signaling pathways, protein metabolism, transcription, photosynthetic rates, and cell elongation (Lambers et al. 1998). The overall effect is that leaf expansion rates are decreased, reducing plant growth. The effect of salt stress on leaf growth has been found to be very rapid and reversible, suggesting that the reduced growth rate is due to osmotic stress rather than the toxic effects of Na^+ and Cl^- accumulation (Munns 2002). Osmotic stress may be the most detrimental short-term effect of salinity (Munns and Termat 1986).

2.2.2 *Ionic Effects*

Saline soil often results in the accumulation of Na^+ and Cl^- ions in plant tissues as they are taken up with water. Although ion toxicity does not occur as rapidly as osmotic stress, the effect on cell and plant metabolism can be severe. Sodium and Cl^- accumulation in plant tissue impacts a number of plant processes including plant growth, metabolism, enzyme activity, membrane integrity, nutrient uptake, and normal functioning of plant metabolism (Greenway and Munns 1980; Volkmar et al. 1998). Fully expanded leaves are typically affected before young leaves because they have been transpiring for longer periods of time and thus have accumulated higher concentrations of ions (Greenway and Munns 1980).

Ion accumulation in the cytosol has been found to affect cytoplasmic metabolism, particularly enzyme activity (Munns 2002). Sodium accumulation interferes with enzyme cationic binding sites and disrupts the hydrophobic electrostatic forces that maintain enzyme structure (Wyn-Jones and Pollard 1983). Sodium and Cl^- ions cause membrane changes resulting in decreased stability, increased fluidity, and increased permeability which alters membrane transport activity (Borochov-Neori and Borochov 1991). Membrane damage results in the influx of Na^+ and Cl^- which further contribute to ion toxicity (Allen et al. 1994). The increased influx of Na^+ and Cl^- ions can also disrupt nutrient uptake by competing with required ions, such as K^+, for uptake with membrane transport proteins, and contribute to nutrient deficiency of salt stressed plants (Allen et al. 1994; Cramer 1997). Accumulation of ions in the cell wall and extracellular space lowers the osmotic potential of the extracellular space drawing water out of the cell, causing turgor loss and cell death (Cramer 1997). In rice (*Oryza sativa*), a salt sensitive species, exposure to NaCl resulted in cellular dehydration due to ion accumulation in the extracellular space (Flowers et al. 1991). Although ionic stress is not as immediate as osmotic stress the effect may be more detrimental particularly in long lived species.

2.2.3 *Effects on the Cell Wall*

During water deficit and salt stress cell wall expansion is often reduced. Changes in the cell wall physical properties, such as cell wall hardening, lower tensile strength, and changes in cell wall composition occur (Iraki et al. 1989a; Iraki et al. 1989b; Neumann et al. 1994; Neumann 1995). As the cell wall plays an important role in regulating growth and turgor pressure, the effect of salt stress on the cell wall will have significant impacts on the plant with regards to growth and water relations. Despite the importance of the cell wall in plant growth and water relations, the effect of salt stress on the cell wall is not well understood.

The primary cell wall is composed of a network of inextensible cellulose microfibrils cross-linked by hemicellulose (also called cross-linking glycans) embedded in a pectin matrix (Carpita 1997; Cosgrove 1999; Buchanan et al. 2000). Cell walls also contain structural proteins, which form networks, and cell wall enzymes. Some plant cells develop lignified secondary cell walls after they have stopped growing. Polysaccharides are the main component of the cell wall as they form the structural network (Buchanan et al. 2000). To understand cell wall structure it is important to study the three main groups of polysaccharides in the cell wall (cellulose, hemicellulose and pectin) as well as one of the main structural components in secondary cell walls (lignin).

Cellulose, the most abundant organic component in plants, is a linear unbranched polysaccharide consisting of 1,4 linked α–glucopyranose units which are arranged into microfibrils (Nobel 1999). Cellulose chains are held in a paracrystalline lattice composed of several dozen (1→4) β-D glucan chains that are hydrogen bonded together creating a structure that has considerable strength (Brett and Waldron 1990). Salt and water stress have been found to affect the cellulose composition of cell walls. In cotton (*Gossypium hirsutum*) primary roots exposed to NaCl, a decrease in the cellulose content of the cell wall was observed (Zhong and Lauchli 1993). In wheat (*Triticum aestivum*) coleoptiles, cellulose increased less in water stressed plants, than non water stressed plants (Wakabayashi et al. 1997). Reduced cellulose synthesis was observed under osmotic stress in cotton (*Gossypium hirsutum*) roots (Zhong and

Lauchli 1988) and tobacco (*Nicotiana tabacum)* cells adapted to osmotic stress (Iraki et al. 1989b). Reduced cellulose synthesis is thought to be a result of decreased cellulose synthase activity due to the displacement of Ca^{2+} from the plasma membrane (Zhong and Lauchli 1988; Carpita 1997). Decreased cellulose synthesis has also been attributed to a reallocation of carbon and energy resources to osmotic adjustment (Singh et al. 1989). Despite the work supporting decreased cellulose in plant cell walls under salt or water stress, there are some contradictory findings. For example, Solomon et al. (1987) found higher amounts of cellulose in the cell walls of pea (*Pisum sativum*) root cultures treated with NaCl.

Hemicellulose is classically described as the fraction of the cell wall that can be extracted with alkaline solutions (Brett and Waldron 1990). This group of polysaccharides is now more accurately referred to as cross-linking glycans (Buchanan et al. 2000). Cross-linking glycans are a group of polysaccharides that hydrogen bond to the cellulose microfibrils linking them together and creating a network. There are two major groups of cross-linking glycans in angiosperms; these include the xyloglucans (XyGs) and the glucuronarabinoxylans (GAXs) (Buchanan et al. 2000). Water and salt stress cause variable responses in the hemicellulose content of plant cell walls. In wheat (*Triticum durum)* leaves under water stress, the hemicellulose content was greater than in the unstressed seedlings; the response varied with cultivar as the drought sensitive cultivar had a greater increase (Rascio et al. 1990). Water stress reduced the increase of hemicellulose in wheat (*Triticum aestivum*) cell walls during growth compared with non-stressed plants (Wakabayashi et al. 1997). In the primary roots of cotton (*Gossypium hirsutum*), NaCl stress caused a decrease in the hemicellulose content compared with untreated seedlings (Zhong and Lauchli 1993).

Pectin is classically described as polysaccharides that are branched, highly hydrated and heterogeneous (Buchanan et al. 2000). Pectin is rich in D-galacturonic acid, rhamnose, arabinose and galactose (Brett and Waldron 1990; Buchanan et al. 2000). There are two main groups of pectin, the homogalacturonans (HGA's) and the rhamnogalacturonans (RGI) (Buchanan et al. 2000). Pectin is thought to have a

number of functions including regulating cell wall porosity, ion balance, cell-cell adhesion, modulating cell wall pH, and functioning as a recognition molecule (Buchanan et al. 2000). Salt and water stress have been found to affect both the composition and amount of pectin in cell walls. In the epicotyls of chick-pea (*Cicer arietinum*), water stress decreased the amount of pectin in the cell walls (Munoz et al. 1993). Pectin extracted from the cell walls of salt adapted tobacco (*Nicotiana tabacum*) were enriched in rhamnose, and the rhamnosyl units of the rhamnogalacturonans were more highly substituted compared to unadapted cells (Iraki et al. 1989b). The increase in rhamnose was thought to be due to a reduction in the average size of the pectin side groups.

Lignin is abundant in secondary cell walls; it is resistant to enzymatic degradation and increases cell wall rigidity (Nobel 1999). Lignin is a complex network of aromatic compounds called phenylpropanoids (Buchanan et al. 2000). The subunits of lignin are formed from 3 aromatic alcohols (Brett and Waldron 1990). Lignin covalently links to cellulose and cross-linking glycan (Buchanan et al. 2000). Lignin generally fills the space in the cell wall that is not already occupied by macromolecules and helps to secure cell wall components preventing plastic expansion (Brett and Waldron 1990). Sclerenchyma, tracheids, and vessel elements are the primary cells in plants that have lignified cell walls (Brett and Waldron 1990). Sodium chloride has been found to cause lignification of the cell wall of tracheary elements in the conducting tissue of pea (*Pisum sativum*) roots (Solomon et al. 1986; Solomon et al. 1987). There are however variable findings, as salt stress does not cause increased lignification in all species. In rice (*Oryza sativa*) NaCl stress reduced cell wall lignin content (Lin and Kao 2001) and in alfalfa (*Medicago sativa*) stems, water stress did not affect cell wall lignification (Deetz et al. 1996).

Not only are the individual cell wall fractions affected by water and salt stress, but the entire cell wall can be altered. In tobacco (*Nicotiana tabacum*) cells adapted to salt stress, the total amount of cell wall material was reduced compared to unadapted cells; this was due primarily to a decrease in the cellulose content of the cell walls (Iraki et al. 1989a). In alfalfa (*Medicago sativa*) stems, water stress reduced the

amount cell wall material compared to unstressed plants (Deetz et al. 1996), while an increase in cell wall material was found in water stressed chick-pea (*Cicer arietinum*) epicotyls (Munoz et al. 1993).

2.3 Mechanisms of Salt Tolerance

Salt stress tolerance requires the ability to tolerate both the osmotic and toxic effects of increased levels of Na^+ and Cl^- (Hagemeyer 1997). The mechanisms of salt tolerance in plants, particularly crop species, have been the focus of numerous studies (Munns 1993). No single mechanism or trait has been found to confer salt tolerance (Munns 1993), but like other stresses, salt stress involves a number of physiological and metabolic processes which are interrelated but difficult to study simultaneously. The need to not only understand how salt affects plants, but also how they are able to tolerate salt stress is critical for selecting species, cultivars or engineering plants that are able to survive in saline areas.

2.3.1 Osmotic Stress Tolerance Mechanisms

Plants have mechanisms that allow them to tolerate the osmotic effects of salinity and although different from drought stress, the drought resistance of a plant has been found to impact its salt tolerance (Redfield and Zwiazek 2000). The ability to withstand drought may be a minimum prerequisite for salt tolerance (Redfield and Zwiazek 2000). Stomatal closure reduces the water requirement, but this is often a short term strategy as transpiration and photosynthesis are also reduced. Water uptake can be maintained through osmotic adjustment, which is the active accumulation of solutes that lower the osmotic potential and thus the plant water potential (Turner and Jones 1980; Fan et al. 1994). Adjustments in cell wall elasticity can also help to maintain water uptake by decreasing the plant water potential through decreases in the pressure potential. Both osmotic and elastic adjustments are considered to be important mechanisms allowing plants to maintain turgor and water uptake under conditions of low soil water potential (Radin 1983; Fan et al. 1994; Volkmar et al. 1998).

2.3.1.1 Osmotic Adjustment

Osmotic adjustment lowers the osmotic potential of a plant, allowing water uptake and turgor maintenance during periods of salt or water stress (Turner and Jones 1980; Radin 1983). Osmotic adjustment facilitates the maintenance of cell elongation, stomatal opening, photosynthesis, and reduces dehydration (Turner and Jones 1980). The capacity for osmotic adjustment varies with species, organ or tissue type, environmental conditions, stress pre-conditioning, the rate of stress development and the degree of stress (Turner and Jones 1980; Morgan 1984).

Osmotic adjustment can occur with the uptake of external ions such as Cl^-, Na^+, or K^+ from the soil solution (Flowers and Yeo 1988) and/or by the increased production of compatible osmolytes (Morgan 1984). Compatible osmolytes are a group of chemically diverse compounds that are highly soluble and do not interfere with metabolism (Buchanan et al. 2000). Some examples of compatible osmolytes include amino acids, tertiary sulfonium compounds, quaternary ammonium compounds, and polyhydric alcohols (Buchanan et al. 2000). Cyclitols accumulate under water or salt stress in maritime pine (*Pinus pinaster*) (Nguyen and Lamant 1988) and ber (*Ziziphus mauritiana)* (Clifford et al. 1998). Other solutes that may contribute include organic acids, such as malate and citrate, (Morgan 1984) glycerol, mannitol, proline and glycinebetaine (Shannon et al. 1994).

In some species soluble carbohydrates are primarily responsible for osmotic adjustment (Zwiazek and Blake 1989). During drought stress, increases in hexose sugars (glucose and fructose) were observed in ber (*Ziziphus mauritiana)* (Clifford et al. 1998). In wheat (*Triticum aestivum*) seedlings the salt tolerant genotypes accumulated higher levels of soluble carbohydrates (glucose, fructose, sucrose and fructan) than the sensitive genotypes (Kerepesi et al. 1998). In jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) exposed to water stress an accumulation of carbohydrates, particularly fructose and glucose, was observed in the roots of both species and in the shoots of white spruce (*Picea glauca*) (Koppenaal et al. 1991). An increase in the total carbohydrates occurred in mature and expanding kenaf (*Hibiscus cannabinus)* leaves under salt stress (Curtis et al. 1988).

Despite the evidence supporting the role soluble carbohydrates play in osmotic adjustment there are some differing views. The accumulation of soluble carbohydrates varies significantly both with genotype and species. In wheat (*Triticum vulgaris*) cv. stork soluble carbohydrate accumulation in both roots and shoots decreased as salinity increased. However, in cv. giza 155 a more salt tolerant cultivar, soluble carbohydrate accumulation increased with increasing salt stress (Hamada and Khulaef 1995). In tomato (*Lycopersicon esculentum)* leaf and root tissue hexose levels decreased while sucrose levels increased, but these changes did not influence osmotic adjustment (Balibrea et al. 1997). It has been suggested that free amino acids are more likely to contribute to osmotic adjustment rather than sugars because during stress stomata close decreasing CO_2 availability (Tyree and Jarvis 1982). As CO_2 levels decrease, a shift occurs from the carboxylation to the oxygenation of ribulose 1-5 bisphosphate, thus increasing the carbon passing through the photosyntheic carbon oxidation cycle, and decreasing the carbon passing through the photosynthetic carbon reduction cycle (Calvin cycle) (Tyree and Jarvis 1982). This shift in metabolism favoring photorespiration would decrease carbohydrate production and increase the substrate available for amino acid production as nitrogen is released in the form of ammonium.

2.3.1.2 Elastic Adjustment

Cell wall elasticity determines the relationship between the relative water content and the turgor pressure of a cell. Changes in cell wall elasticity through elastic adjustment have been found to increase the drought and/or salt tolerance of a number of species (Bowman and Roberts 1985; Blake et al. 1991). The implications of elastic adjustment are not yet fully understood and both increased and decreased cell wall elasticity have been found to improve stress tolerance.

2.3.1.2.1 Cell Wall Elasticity

Plant cell walls can be both elastic and plastic. Cell wall elasticity is reversible and allows cell walls to stretch when a strain is applied and then return to their pre-strain state (Tyree and Jarvis 1982; Nobel 1999). Plasticity is the ability of the cell wall to stretch without returning to its original pre-strain state (Salisbury and Ross

1991). Extensibility is the ability of a cell wall to expand during growth and involves both elastic and plastic properties (Salsbury and Ross 1991). During cell wall expansion the permanent rearrangement and cutting of matrix polymers occurs allowing for a controlled spreading of the cellulose and matrix networks (Cosgrove 1999; Peltier and Marigo 1999). The cell walls of mature cells are not extensible but can be elastic (Taiz 1984). Cell walls are flexible enough to maintain turgor as cell water content fluctuates and the elastic contraction of cell walls is considered to be a passive process that allows cells to shrink during water deficit (Neumann 1995).

Reversible elastic properties are described by Young's modulus (Eq. 2.1) which is the ratio of an applied stress (force/unit area) to the resulting strain (fraction change in length) (Nobel 1999). Large values of Young's modulus indicate that a large stress must be applied to produce a noticeable strain. The stress on plant cell walls is often caused by the hydrostatic pressure within the cell (Nobel 1999).

Young's modulus (force/unit area) = stress/strain Eq. 2.1

Cell wall elasticity, or the volumetric elastic modulus (ε), can be calculated from the change in pressure that occurs for a given change in volume. Higher ε values indicate cells with rigid cell walls. Cells with rigid cell walls have a greater change in pressure for a given change in volume compared with cell walls that have a lower ε value (Tyree and Jarvis 1982; Nobel 1999). Values of ε range from 1 to 50 MPa, cells change about 0.2-10% in volume for each 0.1 MPa change in turgor pressure (Nobel 1999). The ε can be calculated using pressure-volume (P-V) analysis; however only an average value for all tissues and cells can be determined, and it is a relative measure (Neumann 1995). The average bulk modulus of elasticity is calculated from the change in turgor pressure (ΔP) for a given change in relative cell volume ($\Delta V/V$) (Eq. 2.2) (Tyree and Jarvis 1982). Cell wall elasticity varies with both cell volume and turgor pressure (Tyree and Jarvis 1982; Taiz 1984). At lower turgor pressures ε is smaller (cell walls are more elastic) and at higher pressures ε is larger (Tyree and Jarvis 1982).

$$\varepsilon = \Delta P/(\Delta V/V) \qquad \text{Eq. 2.2}$$

Cell wall elasticity is affected by a number of factors. The bulk modulus of cell wall elasticity depends on the structural properties of both the tissue and the cell wall of individual cells (Tyree and Jarvis 1982). Cell wall elasticity may be regulated by changes in cell wall composition (Zwiazek 1991; Renault and Zwiazek 1997), particularly the amount and structure of cell wall polysaccharides (Taiz 1984; Sakurai 1991). Tan and Hogan (1995) suggest that the cell wall matrix cross linking may play an important role in determining cell wall elasticity. Little work has been done examining changes in cell wall composition related to cell wall elasticity under salt stress.

2.3.1.2.2 Changes in Cell Wall Elasticity

Plants with elastic cell walls usually experience little change in turgor pressure as cell water content decreases, as the cell wall is able to 'shrink' around the cell (Evans et al. 1992; Wang and Zwiazek 1999). Elastic cell walls allow the relative water content to decrease to a comparatively low value before turgor is lost, helping to maintain high physiological activity (Radin 1983; Wang and Zwiazek 1999). As water volume decreases solutes are concentrated, decreasing the osmotic potential and facilitating water uptake. Drought stress has been found to increase cell wall elasticity in a number of species including Douglas fir (*Pseudotsuga menziesii*) (Joly and Zaerr 1987), black spruce (*Picea mariana*) (Blake et al. 1991), jack pine (*Pinus banksiana*) (Koppenaal et al. 1991), and lemon (*Citrus limon*) (Ruiz-Sanchez et al. 1997).

Plants with rigid cell walls would have a greater decrease in turgor pressure for a given decrease in water volume, thus lowering the water potential of the plant facilitating water uptake (Bowman and Roberts 1985; Marshall and Dumbroff 1999). Decreased cell wall elasticity during water stress in chaparral shrubs (*Arctostaphylos glandulosa, Quercus dumosa and Ceanothus greggii*) (Bowman and Roberts 1985), white spruce (Marshall and Dumbroff 1999), and ber (*Ziziphus mauritiana*) (Clifford et al. 1998) increased stress tolerance. Under salt stress increased cell wall rigidity

also increased tolerance in mangrove (*Avicennia germinans*) (Suarez and Sobrado 2000), Acacia (*Acacia nilotica*) (Nabil and Coudret 1995), and in alligator weed (*Alternanthera philoxeroides*) (Bolaños and Longstreth 1984).

The benefits of elastic adjustment to the plant during stress tolerance are controversial and not fully understood. The benefits of changes in cell wall elasticity under osmotic stress depend on what is important for the water status of the plant (Shulte 1992). More elastic cell walls are beneficial when the maintenance of turgor pressure is important but not water content, whereas rigid cell walls are beneficial when increased water uptake and water content are considered to be more important than turgor pressure (Shulte 1992).

2.3.2 Ionic Stress Tolerance Mechanisms

The primary mechanisms of ionic stress tolerance are restricting salt entry into the plant and reducing the salt concentration in the cytoplasm (Munns 2002). Ion influx can be controlled by regulating ion movement into the xylem stream. Although not fully understood, it is believed that most control occurs at the suberized endodermis where ions must travel via the symplastic pathway (Greenway and Munns 1980; Hasegawa et al. 2000). Preferential xylem loading and the removal of ions from the upper part of the xylem in the roots may also help to reduce ion accumulation in the shoots (Munns 2002). Ion exclusion is particularly important for perennial woody species as their leaves often live for a year or longer (Allen et al. 1994; Munns 2002). Differences in the salt tolerance of two olive tree (*Olea europaea*) cultivars was thought to be due to their ability to exclude Na^+ and Cl^- ions from leaf tissue (Gucci and Tattini 1997).

All plants growing in saline conditions will eventually accumulate ions and must be able to reduce the accumulation of Na^+ and Cl^- in the cytoplasm. Ions are generally sequestered in the vacuole where they will have little impact on cytoplasmic metabolism and can help to lower the osmotic potential (Apse et al. 1999). Sodium ions are actively transported through the tonoplast into the vacuole through a Na^+/H^+ antiporter in some species such as beets (*Beta vulgaris*) (Blumwald 1987) and Arabidopsis (*Arabidopsis thaliana*) (Apse et al. 1999). In radiata pine (*Pinus radiata*)

ions not only accumulate in the vacuole, but they also accumulate in the ray cells, and in the lumen and cell wall of tracheids (Foster and Sands 1997). When Na^+ and Cl^- are sequestered into the vacuole, osmotica must accumulate in the cytoplasm to balance the osmotic pressure of the ions in the vacuole but not impact cytoplasmic metabolism (Munns 2002).

The compartmentalization of ions within the vacuole is only a limited strategy as the vacuole cannot sequester ions indefinitely (Cheeseman 1988). There are a number of other mechanisms that plants employ to reduce the effect of ion accumulation on cytoplasmic activity. The concentration of Na^+ and Cl^- can be diluted by increased water uptake, and cell expansion provides more vacuole space to sequester ions. This tolerance mechanism is called succulence. A number of halophytes have salt glands or bladders that allow the plant to excrete Na^+ and Cl^- from their leaves (Hasegawa et al. 2000). Older leaves may serve as ion sinks as they will accumulate ions faster due to increased transpiration, later dehiscing and removing those ions from the plant (Hasegawa et al. 2000, Munns 2002). Although the accumulation of ions within the cytoplasm is considered very detrimental to the plant, the cell cytoplasm may contain factors which help to reduce the effect of ions on enzyme activity (Flowers et al. 1986; Hasegawa et al. 2000). The substrate of the cytoplasm is only able to help protect enzymes at low ion concentrations, and as ions accumulate enzyme activity is altered. Tolerance to ionic stress is very important for long-term survival under saline conditions.

2.4 The Biology of *Cornus stolonifera*

Cornus stolonifera (Michx.) syn. *Cornus sericea* L. (red-osier dogwood) has been found to be relatively salt tolerant compared with other species of the boreal forest (Renault et al. 2000). *Cornus stolonifera* is a shrub growing 1-6 m tall; it has clustered stems, small white flowers and dull white fruits (Crane 1989). *Cornus stolonifera* has a very large natural range that extends from Newfoundland to Alaska, south to New Mexico and Arizona, west to California and east to Nebraska (Stephans 1973; Crane 1989). *Cornus stolonifera* prefers moist habitats in riparian areas or in meadows. *Cornus stolonifera* is found in open and wooded areas. It grows in both

rocky and good soil and is very tolerant of flooding (Stephans 1973). A number of birds and animals use *C. stolonifera* for food and cover. These include white-tailed deer, mule deer, elk, moose, mountain goats, cottontail rabbits, snow shoe hares, bobwhite, ring-necked pheasant, wild turkey and others (Crane 1989). By providing shelter and food, particularly in the winter, *C. stolonifera* is an attractive species for reclamation. *Cornus stolonifera* has been recommended for the rehabilitation of sites within its range as it is excellent at stabilizing soil, grows rapidly and is relatively easy to establish (Crane 1989). *Cornus stolonifera* may be a successful species for the reclamation of mining areas (Renault et al. 2000; Renault et al. 2001a).

2.4.1 Cold Acclimation in Cornus stolonifera

Cornus stolonifera is able to tolerate extremely cold temperatures. It tolerated temperatures as low as -196 °C when tested in the laboratory (Newton and Goodin 1989). Stress tolerance may be affected by cold acclimation, which is the process through which plants become more cold tolerant. It has been suggested that the process of cold acclimation, or "hardening off" as it is often referred to in forestry, may affect subsequent stress tolerance, but this has not been well studied (Lang 1996). Different stimuli trigger cold acclimation in different species; however climate conditions such as declining temperature, photoperiod and water availability are the main triggers (Lang 1996). Plants that have gone through cold acclimation often become dormant, lacking visible growth (Lang 1996). During dormancy a plant is usually at its maximum stress tolerance, but during bud break stress tolerance often decreases (Lang 1996). During cold acclimation plants undergo a number of biochemical and physiological changes (Chen et al. 1977). Although a lot is known about the changes that occur during cold acclimation, the effect that these changes have on subsequent stress tolerance during bud break is not known.

When *C. stolonifera* is exposed to short days during acclimation, stomatal resistance initially decreases as transpiration increases (Parsons 1978). The ability to uptake water is decreased yet water loss is increased at the beginning of acclimation thus partially dehydrating the plant and reducing the stem water content even when water is readily available (McKenzie et al. 1974; Parsons 1978). After about 30 to 40

days of acclimation, stomatal resistance increases and the stomata close (Parsons 1978), thus reducing the water requirement of dormant *C. stolonifera*. Water stress and dormancy are closely connected in *C. stolonifera* as short days and water stress were found to increase freezing tolerance compared to seedlings exposed only to short days (Chen et al. 1977). Water stress alone increased freezing tolerance to levels comparable to seedlings experiencing both water stress and short day treatment (Chen et al. 1977).

During the process of cold acclimation the concentration of soluble carbohydrates in *C. stolonifera* stems and roots increases in the fall as starch is broken down, is the highest in midwinter, and then decreases in early spring during bud break (Ashworth et al. 1993; Cappiello and Kling 1994). In *C. stolonifera* a 24-kDa protein related to the dehydrin family begins to accumulate in late August reaching maximum levels midwinter (Sarnighausen et al. 2002). These metabolic changes associated with cold acclimation may affect the stress tolerance of *C. stolonifera*, possibly increasing or decreasing stress tolerance. Little research has been done on the effect of cold acclimation on tolerance to salt stress.

2.4.2 Cornus stolonifera and Salt Stress

The effect of tailings on germination, survival, growth (Renault et al. 1998), gas exchange (Renault et al. 2000), ion accumulation, water potential, photosynthetic rates, and chlorophyll content (Renault et al. 2001a) of *C. stolonifera* seedlings have been examined. Compared to other plant species native of the boreal forest, *C. stolonifera* had a much higher stress tolerance than strawberry (*Fragaria virginiana*), willow (*Salix amygdaloides*), aspen (*Populus tremuloides*) (Renault et al. 1998), raspberry (*Rubus idaeus*), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*) (Renault et al. 2000). When three month old *C. stolonifera* were treated with CT (composite tailings) the shoot dry weight was significantly reduced, while root dry weight was not affected (Renault et al. 2001a). Leaf chlorophyll content and photosynthetic rates decreased when plants were treated with CT (Renault et al. 2001a).

Preliminary work examining the effect of NaCl stress on *C. stolonifera* seedlings suggests germination is inhibited by 100 mM NaCl and reduced at lower concentrations (Renault, personal communication). Despite the sensitivity of germination to salt, three month old *C. stolonifera* seedlings are relatively salt tolerant as treatment with 50 mM NaCl did not affect survival over a four week period (Renault et al. 2001b). *Cornus stolonifera*, like most plant species, has limits to the level of salt stress that it is able to tolerate. When treated with 100 mM NaCl only 30% of the three month old seedlings survived (Renault et al. 2001b). Shoot growth was affected by salt stress more than root growth (Renault et al. 2001b). Salt stress did not reduce leaf chlorophyll content, but did decrease net photosynthetic rates (Renault et al. 2001b).

Previous studies have shown that *C. stolonifera* may restrict the movement of Na^+ and Cl^- ions from the roots into the shoots, thus reducing the effect of ion toxicity (Renault et al. 2001a). The ability of *C. stolonifera* to restrict the movement of ions appears to decrease as the level of salt stress increases. When treated with NaCl at concentrations lower than 100 mM, ion accumulation was greater in the roots than the shoots, while when treated with 100 mM NaCl, Cl^- ions accumulated in the shoots more than the roots (Renault et al. 2001b). Treatment with CT water that contains moderate levels of NaCl (15-20 mM) resulted in higher accumulations of Na^+ and Cl^- ions in the roots than in the shoots of three month old *C. stolonifera* (Renault et al. 2001a). Although *C. stolonifera* may have mechanisms to reduce the effect of ionic stress, it must also have tolerance mechanisms to reduce the effects of osmotic stress to survive in relatively saline soils.

The effect of saline CT on the water relations of *C. stolonifera* has also been examined. Reduced transpiration rates occurred in both three and twelve month old *C. stolonifera* seedlings after CT treatment (Renault et al. 1999; Renault et al. 2001a). Reduced transpiration can be attributed to partial stomatal closure and/or reduced water uptake (Hagemeyer 1997). The rapid reduction of transpiration rates in *C. stolonifera* may limit initial ion accumulation (Renault et al. 1999); however it may also decrease water requirements reducing the osmotic stress on the plant. The effect

of salt stress on the shoot water potential of *C. stolonifera* is variable, but is not affected by plant developmental stage. Shoot water potentials were not significantly affected by salt stress when two and twelve month old seedlings were treated hydroponically with CT water with salt additions (Renault et al. 1998; Renault et al. 1999). This was not supported by more recent work with three month old seedlings as CT treatment increased shoot water potential (Renault et al. 2001a), and 100 mM NaCl decreased shoot water potential (Renault et al. 2001b). The reason for the variable effect of salt stress on the shoot water potential is not known and more research is required in this area to fully understand the effects of NaCl stress of *C. stolonifera*.

2.5 Synthesis

Salt stress is complex as it affects numerous plant processes and is often difficult to determine if observed changes are tolerance mechanisms or stress symptoms; therefore studying salt stress is challenging. Salt stress causes osmotic stress due to increased levels of ions in the soil making water uptake difficult. Salt stress also causes ion toxicity when Na^+ and Cl^- accumulate in plant tissues damaging membranes, affecting nutrient uptake and disrupting enzyme activity. Both osmotic stress and ion toxicity can affect the cell wall reducing cell wall expansion, altering physical properties and affecting composition. Tolerance to salt stress requires mechanisms to reduce both the effects of osmotic stress and ion toxicity. Osmotic stress tolerance mechanisms include osmotic and elastic adjustment. Alterations in cell wall elasticity can increase the salt tolerance of a plant. Increased cell wall elasticity allows the plant to maintain turgor at lower water volumes. Decreased cell wall elasticity decreases the pressure potential facilitating water uptake. Ionic stress tolerance mechanisms involve restricting the movement of ions to sensitive tissues, sequestering ions in non-sensitive areas, such as the vacuole, and/or excreting ions. Plant life history and developmental stage can affect salt tolerance. The physiological, biochemical and molecular changes that occur during the process of cold acclimation may influence subsequent stress tolerance. Different species vary in their level of

stress tolerance. Previous work identified *C. stolonifera* as a relatively salt tolerant species compared to other plants of the boreal forest. *Cornus stolonifera* has been recommended as a potential species for the reclamation of saline oil sands tailings; however little is known about the salt tolerance mechanisms in woody plants of the boreal forest.

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3.0 The Effect of NaCl on Actively Growing *Cornus stolonifera* Seedlings

3.1 Introduction

Salt stress causes both osmotic stress due to a decrease in the soil water potential, and ionic stress due to ion accumulation in plant tissues (Greenway and Munns 1980; Cramer 1997). Understanding the effect of NaCl stress on plants is becoming more important as the amount of salt present in soils is rapidly increasing due to secondary salinization. Little is known about the effects of salt stress on woody plants, particularly species of the boreal forest. *Cornus stolonifera* (red-osier dogwood) has been identified as a relatively salt tolerant species compared with other species of the boreal forest (Renault et al. 2000). *Cornus stolonifera* has been recommended for the rehabilitation of degraded sites within its range (Crane 1989), and has been suggested as a good species for use in the reclamation of saline mining areas in northeastern Alberta (Renault et al. 2000; Renault et al. 2001a).

Previous work has shown that *C. stolonifera* is able to restrict the movement of Cl^- and Na^+ to shoot tissue, reducing ion accumulation and ion toxicity (Renault et al. 2001a). To tolerate salt stress *C. stolonifera* must not only have mechanisms to reduce the effects of ionic stress but must also be able to tolerate osmotic stress, which is considered to be the most immediate stress imposed by NaCl (Munns and Termat 1986). Mechanisms to tolerate osmotic stress include osmotic adjustment through the active accumulation of compatible osmolytes within plant cells and adjustments to cell wall elasticity. Adjustments in cell wall elasticity can help to maintain water uptake as more rigid cell walls will help to decrease the pressure potential thus decreasing the plant water potential. More elastic cell walls help to maintain turgor pressure at lower relative water volumes as the cell walls are able to shrink around the cell contents (Bowman and Roberts 1985; Ruiz-Sánchez et al. 1997). To understand the effects of NaCl stress on plant cell walls it is important not only to examine cell wall elasticity, but also cell wall composition. Sodium chloride stress has been found to change the physical cell wall properties of tobacco (*Nicotina*

tobaccum) (Iraki et al. 1989a) and maize (*Zea mays*) (Neumann 1994), and cell wall structural properties of pea (*Pisum sativum*) (Solomon et al. 1987) and cotton (*Gossypium hirsutum*) (Zhong and Lauchli 1993).

Examining growth, photosynthesis and stomatal resistance allows for a general understanding of how the salt stress is affecting the plant. Understanding plant water relations helps to gain knowledge about the water status of the plant and can be used with findings of cell wall elasticity to determine if cell wall elastic adjustments are contributing to increased stress tolerance (Hinckley et al. 1980). The first objective was to examine the effects of NaCl on growth parameters, water potential, osmotic potential at full turgor, osmotic potential at turgor loss, pressure potential at full turgor and relative water content at turgor loss of *C. stolonifera* shoot tissue. It was expected that the salt stress would reduce growth and alter water relation parameters, allowing survival under saline conditions. The second objective of this study was to study the effect of NaCl on the cell walls in shoot tissue of actively growing *C. stolonifera* seedlings. It was expected that the salt stress would alter both cell wall elasticity and cell wall composition and that these changes would increase the salt tolerance of *C. stolonifera*.

3.2 Material and Methods

3.2.1 Plant Material

Cornus stolonifera seeds were collected from north of Fort McMurray, Alberta, Canada (57°01.67 N, 111°30.6 W). Seeds were wrapped in moist paper towel, sealed in plastic bags and placed at 4°C for 5 weeks to stratify the seeds. After stratification seeds were washed under running water for 24 hours and then planted in a mixture of peat moss and sand (3:1, by volume), in a planting tray (72 Pro-tray, cell depth 6 cm, diameter at top 3.9 cm). The seedlings were grown in the greenhouse for 3 months under the following conditions: day temperature was 25 ± 3°C and the night temperature was 17 ± 2°C, the relative humidity was approximately 60%, the photoperiod was 16 hours, ambient light was supplemented with 400 W high pressure sodium lamps (P.L. Light Systems, Beamsville, Ontario, Canada).

3.2.2 Stress Treatments

Three month old *C. stolonifera* seedlings were transferred to a hydroponic system (Figure 3.1). The roots were washed free of sand and peat and the seedlings were fitted with small foam stoppers at their root collars. Fifteen seedlings were inserted into holes in the lid of a 10 liter plastic container. The container was filled with half strength modified Hogland's solution, containing 0.5 mM KH_2PO_4, 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1.0 mM $MgSO_4$, 23.30 μM H_3BO_3, 12.78 μM $MnCl_2 \cdot 4H_2O$, 6.05 μM $ZnSO_4 \cdot 7H_2O$, 8.03 μM $CuSO_4 \cdot 5H_2O$, 0.08 μM MoO_3, 6.80 μM Fe-EDTA (Renault et al. 2001a). The solution was aerated using aquarium pumps (Maxima, Montreal, Air output 2500 cm^3 min^{-1}). The nutrient solution was changed weekly for the first 3 weeks of the experiment while the seedlings were small and then the solution was changed every 3 to 4 days during week 4 and 5 to prevent nutrient deficiency. The containers and pumps were randomly moved each solution change to ensure even growing conditions for all seedlings. Deionized water was added between solution changes to maintain 10 liters of solution in each container.

Cornus stolonifera seedlings were allowed to acclimate in the nutrient solution for one week prior to treatment. Three treatments of 0, 25 or 50 mM NaCl were replicated three times. Sodium chloride was added to the nutrient solution when the solutions were changed one week after the seedlings were transferred to the hydroponic system. The conductivity of the control solution was 1.07 mS cm^{-1}, 3.57 mS cm^{-1} for the 25 mM NaCl solution and 6.11 mS cm^{-1} for the 50 mM NaCl solution. The calculated osmotic potentials of the nutrient solutions were -0.03 MPa, -0.16 MPa and -0.28 MPa for the control, 25 mM and 50 mM solutions respectively. The pH of the solutions was approximately 5.0. Two experiments were run following the above protocol. The preliminary experiment examined cell wall elasticity after a period of 24 hours; the second experiment was used for all other measurements.

Figure 3.1 Hydroponic system in the greenhouse.

3.2.3 *Growth*

Seedling shoot height was measured zero, 14 and 28 days after the beginning of treatment. Height was determined as the distance from the root collar to the tip of the newest pair of leaves. Shoot and root dry weights were determined after the tissue was lyophilized with a freeze dry system (Model 77520, Labconco Co. Kansas City, MO, USA). After 7, 21, 28 and 35 days of treatment seedlings were randomly selected to determine dry weight. The roots were washed, and then roots and shoots were separated and frozen in liquid nitrogen. Samples were stored at -80°C until lyophilization. The root/shoot dry weight ratio was also calculated.

3.2.4 *Water Potential*

Twenty-eight days after the beginning of treatment, three seedlings per treatment per replicate were randomly selected to determine midday shoot water potential using a pressure chamber operated at 0.02 MPa s^{-2} (model 1000, PMS Instruments, Corvallis, Oregon, USA). The top 6 to 10 cm of the shoot was cut and sealed within the pressure chamber with the cut end protruding. The shoot water potential was recorded when xylem sap was first observed on the cut end (Scholander et al. 1965).

3.2.5 *Photosynthesis, Pigment Analysis and Transpiration*

The amount of chlorophyll a, chlorophyll b, total chlorophyll (a and b), the ratio of chlorophyll a/ chlorophyll b and the amount of carotenoids in leaves of *C. stolonifera* seedlings were determined after 32 days of treatment. Eight leaf disks (6 mm in diameter) were cut with a cork borer from the newest mature leaf of 9 plants per treatment. Major veins were avoided when leaf disks were cut. Four disks from each plant were placed in a scintillation vial, samples were replicated twice and final values were averaged. The fresh mass of the four leaf disks was recorded. Two milliliters of 80% acetone was added to each scintillation vial. Samples were covered in tin foil and incubated in the dark, to prevent chlorophyll degradation, on a shaker

for 4 hours. The acetone containing the pigments was removed and placed in a new scintillation vial. An additional 2 ml of 80% acetone was added to each sample, and the samples were then incubated overnight in the dark on a shaker to remove any remaining pigments. The two acetone washes were combined and the absorbance was read at 480, 645 and 663 nm using an UV/Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). Equations from Šesták et al. (1971) based on the original paper by MacKinney (1941) were used to calculate the amount of chlorophyll a (Eq. 3.1), b (Eq. 3.2) and total chlorophyll (Eq. 3.3). The ratio of chlorophyll a/ chlorophyll b was also calculated. The amounts of carotenoids were quantified using an equation (Eq. 3.4) from Davies (1976). The formulae used determined the pigment amount in mg L^{-1}; values were then converted to mg g^{-1} fresh weight.

$$\text{Chl a} = 12.72(A663) - 2.58(A645) \quad \text{Eq. 3.1}$$

$$\text{Chl b} = 22.87(A645) - 4.67(A663) \quad \text{Eq. 3.2}$$

$$\text{Chl a and b (total chlorophyll)} = 8.05(A663) + 20.29(A645) \quad \text{Eq. 3.3}$$

$$\text{Carotenoids} = A480 + 0.114(A663) - 0.638(A645) \quad \text{Eq. 3.4}$$

Photosynthesis was determined using an Infra Red Gas Analyzer (IRGA) (Qubit Systems Inc. Cambridge, England). Measurements were done between 10:00 am and 2:30 pm. Three seedlings per treatment per replicate were randomly selected and a fully mature leaf near the top of each seedling was removed and sealed within the IRGA leaf chamber. Photosynthetic rates were determined at 400 µmoles photons m^{-2} s^{-1} (Quantum/radiometer/photometer, LI-COR Inc., Lincoln, Nebraska, USA). The difference in carbon dioxide (dCO_2), measured in µmoles l^{-1}, was found by subtracting the amount of CO_2 entering the leaf chamber from the amount of CO_2 exiting the leaf chamber. The flow rate of CO_2 into the leaf chamber was 0.001 l s^{-1}. The CO_2 exchange rate (µmoles s^{-1}) was calculated by multiplying dCO_2 by the flow rate (Eq. 3.5), which was then multiplied by the leaf area (m^2) to determine the photosynthetic rate in µmoles of CO_2 m^{-2} s^{-1}.

photosynthetic rate = (dCO_2)(flow rate)(leaf area) Eq. 3.5

Twenty eight days after the beginning of treatment, transpiration and stomatal conductance (1/stomatal resistance) were determined using one of the leaves from the third pair from the top of the seedling. Measurements were done on 9 seedlings per treatment, in the greenhouse, at a light intensity of approximately 150 μmoles m^{-2} s^{-1}, between 10:00 am and 2:00 pm using a steady state porometer (LI-1600, LI-COR, Inc., Lincon Nebraska, USA).

3.2.6 Soluble Carbohydrates

Soluble carbohydrate extraction was done following the method outlined by Zwiazek (1991). After 21 days of treatment three seedlings per treatment per replicate were randomly selected, the roots were rinsed with deionized water. Shoots and roots were then separated at the root collar and frozen in liquid nitrogen. The samples were stored at -80°C prior to lyophilization. Freeze dried tissues were ground using a Wiley mill (Arthur H. Thomas Co., Philadelphia, USA) with a 40 mesh screen. Ten milliliters of 80% ethanol was added to 50 mg of ground freeze dried plant material and heated at 80°C for 30 min. The ethanol containing the soluble carbohydrates was removed and the tissues were washed with 80% ethanol a second time. The ethanol extracts were then combined and filtered. The ethanol was evaporated using a rotovapour (RE 111, Büchi, Switzerland). Samples were then re-solublized in 25 ml of deionized water.

The Anthrone method originally developed by Dreywood (1946) and then modified by Morris (1948) was used to quantify the soluble carbohydrates as β-D-glucose equivalents. Anthrone reagent (9,10 dihydro-9-oxoanthacene) was prepared with 1% thiourea and 0.1% Anthrone in 76% sulfuric acid; in a test tube 4 ml of Anthrone reagent was added to 0.5 ml of extracted soluble carbohydrates and 0.5 ml of deionized water. The samples were vortexed and heated in a water bath at 100°C for 15 min. During heating the polysaccharides were hydrolyzed to monosaccharides which then dehydrated forming furfural derivatives which reacted with Anthrone to

produce a green colour (Raunkjær et al. 1994). The absorbance of the samples was determined at 620 nm using a UV/Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). For each sample a blank was treated in a similar manner to the samples but 4 ml 76% sulfuric acid was added instead of the Anthrone reagent. The absorbance of the blanks was subtracted from the absorbance of the samples to give a corrected value. The carbohydrate content of the plant tissue was calculated using a standard curve (known glucose concentration vs. absorbance at 620 nm (Figure A1). Final values were expressed as mg of carbohydrates g^{-1} dry weight of tissue (shoot or root).

3.2.7 P-V Measurements

Cell wall elasticity and plant water relations were studied using the well-established pressure-volume (P-V) curve method (Scholander et al. 1965; Tyree and Hammel 1972; Cheung et al. 1975; Hinckley et al. 1980; Nabil and Coudret 1995). Pressure volume curves were constructed for seedlings one, seven and 21 days after treatment using the repeat pressurization method (Parker and Colombo 1995; Clifford et al. 1998). Three seedlings per replicate were randomly selected from each treatment; roots were rinsed and cut from the shoot at the root collar. The roots were immediately frozen in liquid nitrogen. The bottom 2 cm of the cut end of the shoot was submersed in deionized water in a large beaker which was sealed with parafilm. The shoots were rehydrated over night (approximately 17 hours) in the dark at 4°C. It was important to ensure that the samples were fully hydrated when P-V measurements were done as P-V theory is based on the assumption that samples are at full turgor when the first measurement is recorded (Evans et al. 1990). For this reason samples were only used if they were found to have an initial water potential greater than -0.6 MPa.

The following morning the top 6 to 10 cm of the rehydrated shoot was used for P-V analysis. As rapidly as possible after removing the shoot from the sealed container, the shoot was re-cut, and placed in the pressure chamber (model 1000, PMS Instruments, Corvallis, Oregon, USA) with the cut end protruding. Water potential of the shoot was determined when xylem sap was first observed on the cut

end (Scholander et al. 1965). After the water potential was determined at full saturation, the samples were weighed to then determine the turgid weight (TW). Repeat water potential measurements were made over time followed by fresh weight (FW) measurements to calculate the water content of the sample (Parker and Colombo 1995). Shoots were allowed to dehydrate through transpiration while on the laboratory bench. Measurements continued until the shoot broke or the shoot was severely damaged; typically 10 to 20 measurements were made for each sample. Shoot tissue was then lyophilized and dry weight (DW) was determined. Relative water content (RWC) was calculated for each sample using the equation (Eq. 3.6) from Wang and Zwiazek (1999).

$$RWC = (FW-DW)/(TW-DW) \qquad \text{Eq. 3.6}$$

Pressure-volume curves were constructed by plotting -1/water potential (ψ) vs. 1-RWC (Figure 3.2). The positive reciprocal of water potential was plotted so that a linear relationship could be obtained (Hinckley et al. 1980). P-V curves have both a linear and curvilinear portion; the linear portion of the curve was determined with the best fit straight line. The curvilinear portion of the P-V curve was fitted manually. The intersection point between the curvilinear and linear portion of the P-V curve is the point of turgor loss (Cheung et al. 1975). Extrapolation of the linear portion of the line to the y-axis gives the osmotic potential of the sample at full turgor ($\psi_{\pi 100}$) (Figure 3.2) and extrapolation to the x-axis gives the apoplastic water content (AWC). Using the point of turgor loss, both the relative water content at turgor loss (RWC_0) and the pressure potential at turgor loss (ψ_{P0}) can be determined (Figure 3.2). The pressure potential at full turgor (ψ_{P100}) can be calculated from the difference between the water potential at full turgor (ψ_{100}) and the osmotic potential at full turgor ($\psi_{\pi 100}$) (Figure 3.2). The bulk modulus of cell wall elasticity (ε) can be calculated using the equation (Eq. 3.7) from Nabil and Coudret (1995).

$$\varepsilon = (\psi_{P100} - \psi_{P0})(1-AWC)/(1-RWC_0) \qquad \text{Eq. 3.7}$$

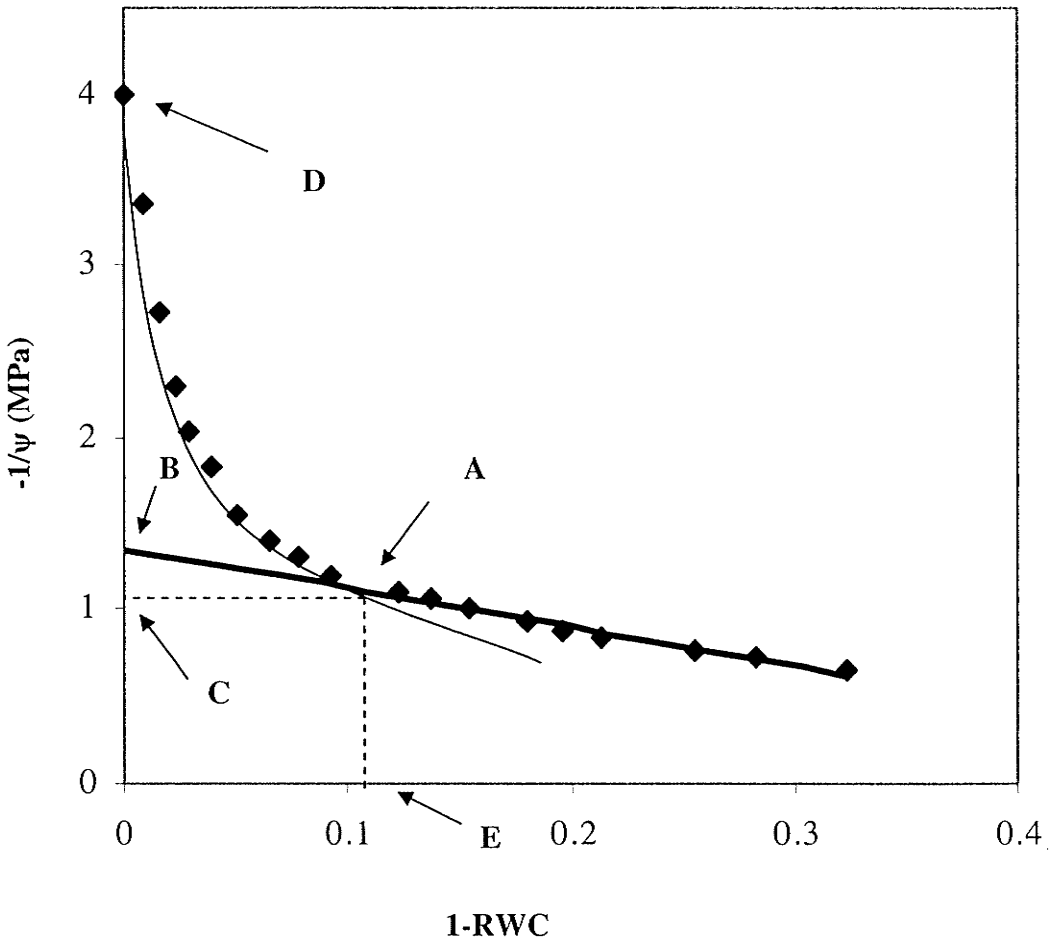

Figure 3.2 Pressure-volume (P-V) curve used to determine water relation parameters: A, point of turgor loss; B, osmotic potential at full turgor ($\psi_{\pi 100}$); C, osmotic potential at turgor loss ($\psi_{\pi 0}$); D, water potential at full turgor (ψ_{100}); D-B, pressure potential at full turgor ($\psi_{P100} = \psi_{100} - \psi_{\pi 100}$); and E, relative water content at turgor loss (RWC_0).

The parameters that were examined from P-V curves in this study included: ε, $\psi_{\pi 100}$, $\psi_{\pi 0}$, ψ_{P100}, and RWC_0. Apoplastic water content was not examined as it was found to give unrealistic values for *C. stolonifera*. Barker et al. (1993) found similar problems with the AWC determined from pressure volume curves in 5 different species of forage grasses, as the AWC was found to range from -10 to 90%.

3.2.8 Cell Wall Separation

3.2.8.1 Cell Wall Extraction

Plants harvested for soluble carbohydrate analysis were also used for cell wall analysis; the ground freeze-dried plant tissue was prepared as described in section 3.2.7. The cell walls were extracted from the ground shoot tissue following the protocol described by Zwiazek (1991). Figure 3.3 outlines the cell wall extraction steps.

Step 1: Six hundred milligrams of freeze dried shoot tissue was weighed into a 30 ml centrifuge test tube.

Step 2: Fifteen milliliters of 85% ethanol was added to each sample and then heated at 80°C for 30 min. The ethanol was removed, and the samples were again washed with 15 ml of 85% ethanol. The samples were then washed twice with 10 ml of deionized water to remove the remaining ethanol. After each addition of water each sample was vortexed, centrifuged (4900 *g*) and the supernatant discarded. The remaining shoot tissue was lyophilized.

Step 3: Seven milliliters of ice cold 50 mM tris buffer (pH 7.2) containing 10 mM ascorbate was added to the dry samples, vortexed, centrifuged (4900 *g*) and then the supernatant was removed.

Step 4: Ten milliliters of 500 mM potassium-phosphate buffer (pH 7.0) was added to each sample, vortexed, centrifuged (4900 *g*) and then the supernatant was removed. The samples were then washed a second time with potassium-phosphate buffer and four times with deionized water following the procedure described above. The remaining samples were then lyophilized.

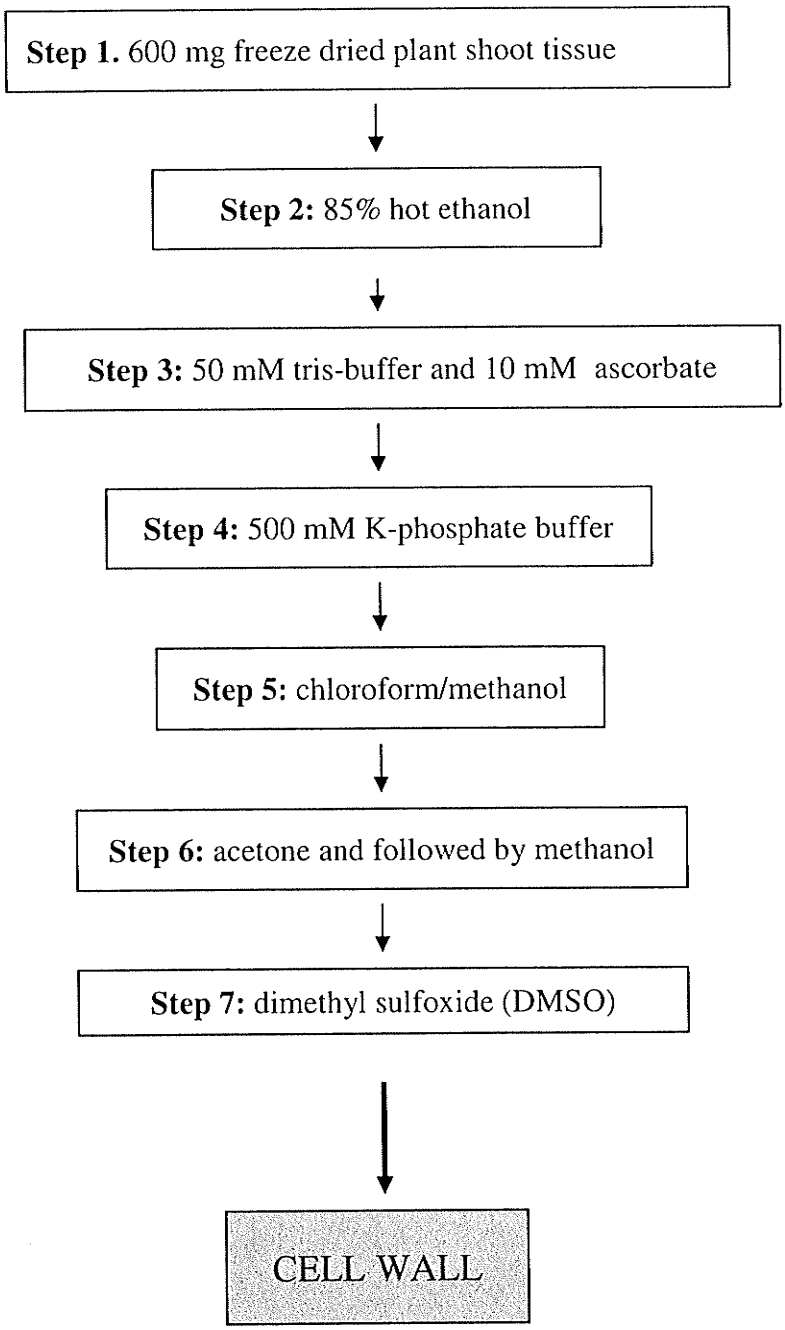

Figure 3.3 Procedure for cell wall extraction from shoot tissue of *C. stolonifera*.

Step 5: Ten milliliters of chloroform and methanol (equal volumes) were added to each sample and heated at 45°C for 30 min. The chloroform and methanol was then removed and the chloroform and methanol wash was repeated.

Step 6: Ten milliliters of acetone was added to each sample, centrifuged (4900 *g*) and the supernatant was discarded. Then, 10 ml of methanol was added twice to each sample; after centrifugation (4900 *g*) the supernatant was discarded. The samples were washed three times with deionized water following the procedure above. The remaining tissue was lyophilized.

Step 7: Twenty milliliters of dimethyl sulfoxide (DMSO) was added to each sample; they were sealed with parafilm, then shaken vigorously and placed on a shaker for 24 hours. The DMSO was removed and discarded. The wash with DMSO was repeated twice to ensure all starch was removed. Samples, now containing only cell wall material, were washed three times with deionized water as described above and then lyophilized. The weight of the lyophilized cell wall material was determined.

3.2.8.2 Separation of Cell Wall Fractions

One hundred and fifty milligrams of freeze-dried cell wall material was used from each sample to separate the cell wall into a pectin, hemicellulose and cellulose fraction. Lignin was removed but not collected, as it was analyzed separately. Figure 3.4 outlines steps followed for the separation of the cell wall fractions. To separate the pectin fraction, 20 ml of 0.5% ammonium oxalate was added to each sample. The samples were vortexed and heated at 100°C for 1 hour in a water bath. After centrifugation (4900 *g*) the supernatant was collected, and the wash with ammonium oxalate was repeated. The supernatants containing the pectin fraction were combined and filtered through a whatman GF/F glass microfiber filter under vacuum. Samples were dialyzed using 3500 MWCO Spectra/Por membranes (flat width 45 mm, diameter 29 mm, vol/length 6.4 ml cm^{-1}, Fisher Scientific, Canada) for 18 hours against running water, 4 hours in distilled water and 2 hours in deionized water. The pectin fraction was lyophilized and weighed.

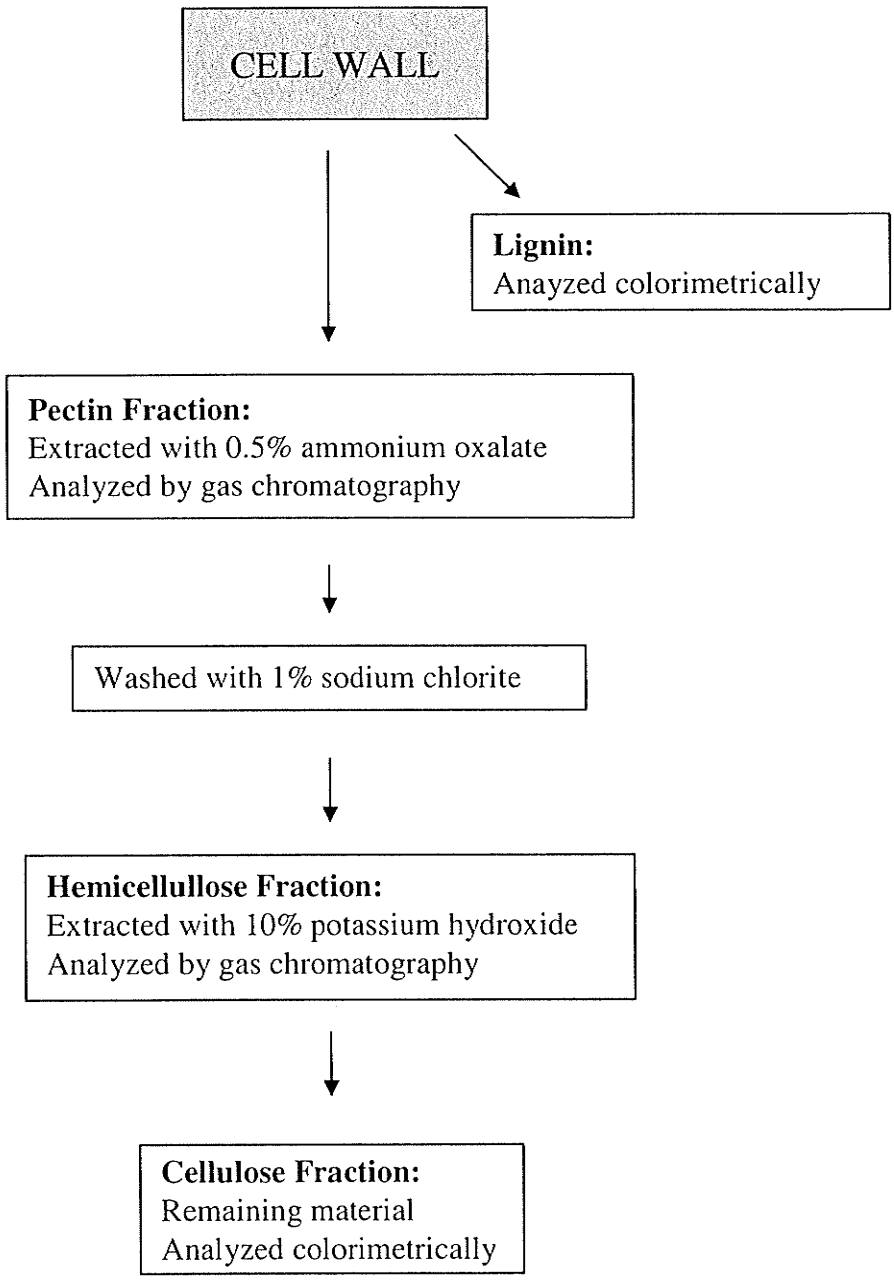

Figure 3.4 Procedure for isolating the polysaccharide fractions (pectin, hemicellulose and cellulose) and lignin from cell walls from *C. stolonifera* shoots.

The remaining cell wall material was washed three times with deionized water following the procedure described for cell wall extraction. Following lyophilization, the remaining cell wall material was washed with 10 ml of 1% sodium chlorite for one hour at room temperature to remove the lignin prior to hemicellulose extraction. The supernatant was removed and discarded. The wash with 1% sodium chlorite was repeated twice to ensure all lignin was removed. The samples were then washed three times with deionized water before lyophilization. The remaining cell wall material was then extracted with 10 ml of 10% potassium hydroxide to extract the hemicellulose fraction. Samples were vortexed and placed on a shaker at room temperature for 48 hours. The supernatant was removed and collected. The samples were washed a second time with 10 ml of 10% potassium hydroxide for 48 hours. The supernatants containing the hemicellulose fraction were combined and filtered through a whatman GF/F glass microfiber filter under vacuum. Samples were then dialyzed using 3500 MWCO Spectra/Por membranes (flat width 45 mm, diameter 29 mm, vol/length 6.4 ml cm-1, Fisher Scientific, Canada) for 18 hours against running water, 4 hours in distilled water and 2 hours in deionized water. The hemicellulose fraction was lyophilized and weighed. The remaining cell wall tissue containing the cellulose fraction was lyophilized and then weighed.

3.2.9 Analysis of Cell Wall Components

3.2.9.1 Pectin and Hemicellulose Fractions

Pectin and hemicellulose fractions were analyzed by gas chromatography (GC). Two milligrams of either pectin or hemicellulose were hydrolyzed with 2 ml of 2 N trifluroacetic acid heated at 121°C sealed in 4 ml vials for three hours. The trifluroacetic acid was then evaporated under a stream of air. To each sample, 300 μl of deionized water was added and samples were vortexed; 200 μl was then transferred to another vial prior to the carbohydrate reduction. Erythritol was used as the internal standard. Ten microliters of 2.5 mg ml^{-1} of erythritol was added to each sample. The carbohydrates were then reduced with 600 μl sodium borohydride (20 mg ml^{-1}) which

was added in 150 μl aliquots. The pH of the samples was 10. The samples then sat overnight for at least 16 hours at room temperature to ensure that the reduction of carbohydrates occurred.

After the carbohydrate reduction 15 drops of 2 N acetic acid was added to each sample to decrease the pH to 4.5. The samples were then dried under a stream of air. The samples were washed with 100 μl deionized water and 1 ml of 1% acetic acid in methanol. After evaporation of the acetic acid and methanol, this step was repeated three times. Under a fume hood the samples were acetylated with 500 μl acetic anhydride, 100 μl pyridine and 500 μl dichloromethane. The samples were shaken vigorously and let sit overnight.

The acetic anhydride and pyridine was removed from the samples with the addition of 1 ml 0.1 N hydrochloric acid. The aqueous phase containing pyridine and acetic anhydride was removed from the samples leaving the pectin or hemicellulose in the dichloromethane. The samples were washed four times with 1 ml of 0.1 N hydrochloric acid. The dichloromethane was evaporated under an air stream. Five hundred microliters of dichloromethane and 100 mg of sodium sulfate (to absorb any remaining water) were added to the samples prior to injection into the gas chromatograph. One microliter of each sample was injected into the gas chromatograph (CP-3800, Varian, Walnut Creek, CA, USA). The column used was a WCOT Fused Silica column (#CP8712, Varian, Walnut Creek, CA, USA). The column length was 30 m, and the inside diameter was 0.25 mm. An initial column temperature of 180°C was held for 30 seconds, and then ramped to 240°C at a rate of 5°C min^{-1}. The column temperature was then held at 240°C for 5 minutes. The detector had a temperature of 250°C. There was a constant flow of helium (1.3 ml min^{-1}) and a split ratio of 20. Identification of the gas chromatograph peaks was done using samples containing known sugars (Figure A2).

3.2.9.2 Cellulose Fraction

The cellulose fraction was analyzed colorimetrically following the Updergraff procedure (Updergraff 1969) to determine the amount of glucose present in this fraction. Three milliliters of acetic nitric reagent (10% nitric acid in 80% acetic acid)

were added to 10 mg of the freeze dried cellulose fraction in a 15 ml centrifuge test tube. The samples were heated at 100°C for 30 minutes. The supernatant containing the acetic acid reagent was removed and discarded. The samples were then washed 2 times with 5 ml of deionized water. To each sample 8 ml of 67% sulfuric acid was added in two 4 ml aliquots, vortexing after each addition. After one hour, 25 ml of deionized water was added to dilute the sample. The Anthrone test (Ashwell 1957; Dreywood 1946) was used to quantify the amount of glucose in the cellulose. Anthrone reagent (9,10 dihydro-9-oxoanthacene) was prepared with 1% thiourea (to stabilized the solution), and 0.1% Anthrone in 76% sulfuric acid. Four milliliters of Anthrone reagent was added to 0.5 ml of the cellulose extract along with 0.5 ml of deionized water. The samples were vortexed and heated for 15 min at 100°C in a water bath. The absorbance was read at 620 nm on a UV/Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The glucose content of the cellulose fraction was calculated using a standard curve (known glucose concentration vs. absorbance at 620 nm) (Appendix 1.1). Final values were expressed as mg of glucose g^{-1} dry weight of cellulose.

3.2.9.3 Lignin Fraction

The amount of lignin in the cell wall was determined colorimetrically from freeze dried cell wall material using the method outlined by Zwiazek (1991), originally developed by Morrison (1972). Nine samples per treatment were used for lignin analysis. Ten milligrams of freeze dried cell wall tissues were placed in scintillation vials and 4 ml of 25 % acetyl bromide in glacial acetic acid was added to each sample. The vials were sealed tightly and heated for 30 minutes in a water bath at 70°C. The samples were then cooled and 4 ml of 2 M sodium hydroxide was added. After 45 minutes 5 ml of the supernatant was removed, taking care not to pipet large amounts of cell wall material, and placed in an Erlenmeyer flask. To the 5 ml of lignin extract, 400 μl of hydroxylamine hydrochloride was added. The lignin extract was diluted by the addition of 50 ml of deionized water. The absorbance of the samples was read at 280 nm. The amount of lignin in g l^{-1} (C) was quantified using an equation (Eq. 3.8) modified from Morrison (1972) and the specific absorbance of 20.9

l g^{-1} at 280 nm was used (Crawford 1981). The final amount of lignin was expressed as percent of cell wall material.

$$C = A280/20.9 \text{ l/g} \qquad \text{Eq. 3.8}$$

3.2.10 Data Analysis

Data was analyzed with a general linear model (GLM) procedure using one way analysis of variance (ANOVA). Means were compared with Duncan's New multiple range test using SPSS (Version 1.0, SPSS Inc., Chicago, USA). Statistically significant differences were determined at the 0.05 level.

3.3 Results

3.3.1 Growth

After 35 days of treatment all control and salt treated *C. stolonifera* seedlings survived and showed substantial shoot growth (Figure 3.5). Seedling height increased throughout the experimental period in all treatments. There was no significant difference in height growth between the 0 and 25 mM NaCl treated seedlings (Figure 3.6). Although seedlings treated with 50 mM NaCl had 8.4% less height increased compared to the controls after 28 days of treatment, this difference was not significantly different (Figure 3.6). Shoot and root dry weights increased throughout the duration of the experiment (Figure 3.7). After 35 days of treatment no significant difference between treatments was found for root dry weight (Figure 3.7a), while seedlings treated with 25 and 50 mM NaCl had a significantly lower shoot dry weights (31.3% and 36.3% respectively), compared to the control seedlings (Figure 3.7b). The effect of NaCl on shoot and root dry weight varied with time indicating the differences between treatments increased throughout the experimental period. Seedlings treated with 25 and 50 mM NaCl had a slightly higher root/shoot dry weight ratios throughout the experimental period compared to the untreated seedlings, but the difference was not significant (Figure 3.7c).

Figure 3.5 *Cornus stolonifera* seedlings after 28 days after treatment; 0 mM NaCl (control) (a), 25 mM NaCl (b), 50 mM NaCl (c).

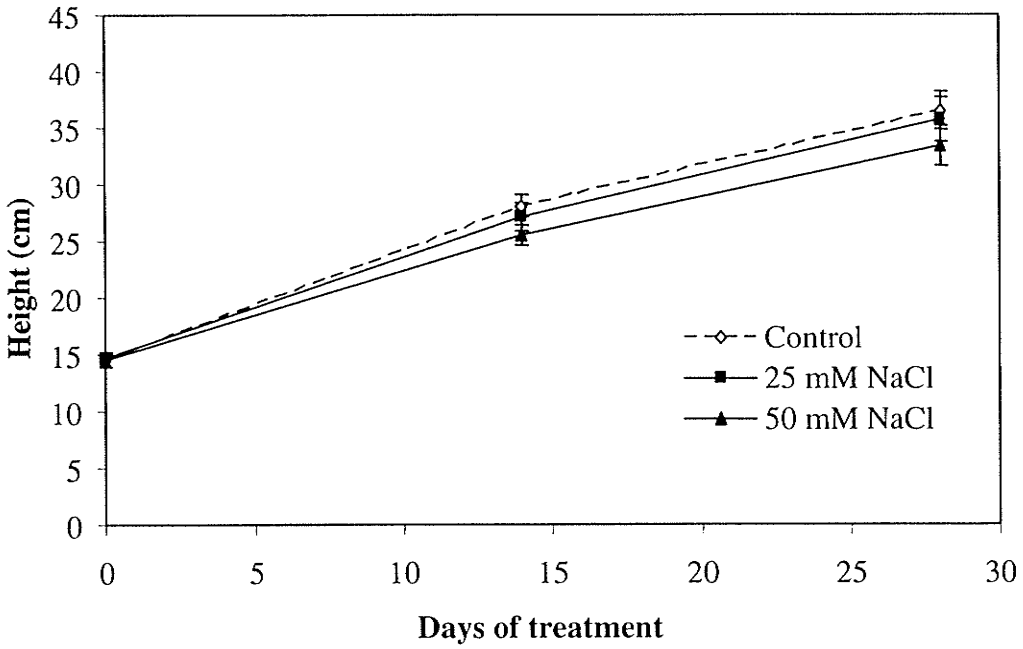

Figure 3.6 *Cornus stolonifera* seedling height, measured after 0 (n = 45), 14 (n = 36) and 28 (n = 15) days of treatment with either 0, 25 or 50 mM NaCl (mean ± SE). No significant differences were found between treatments

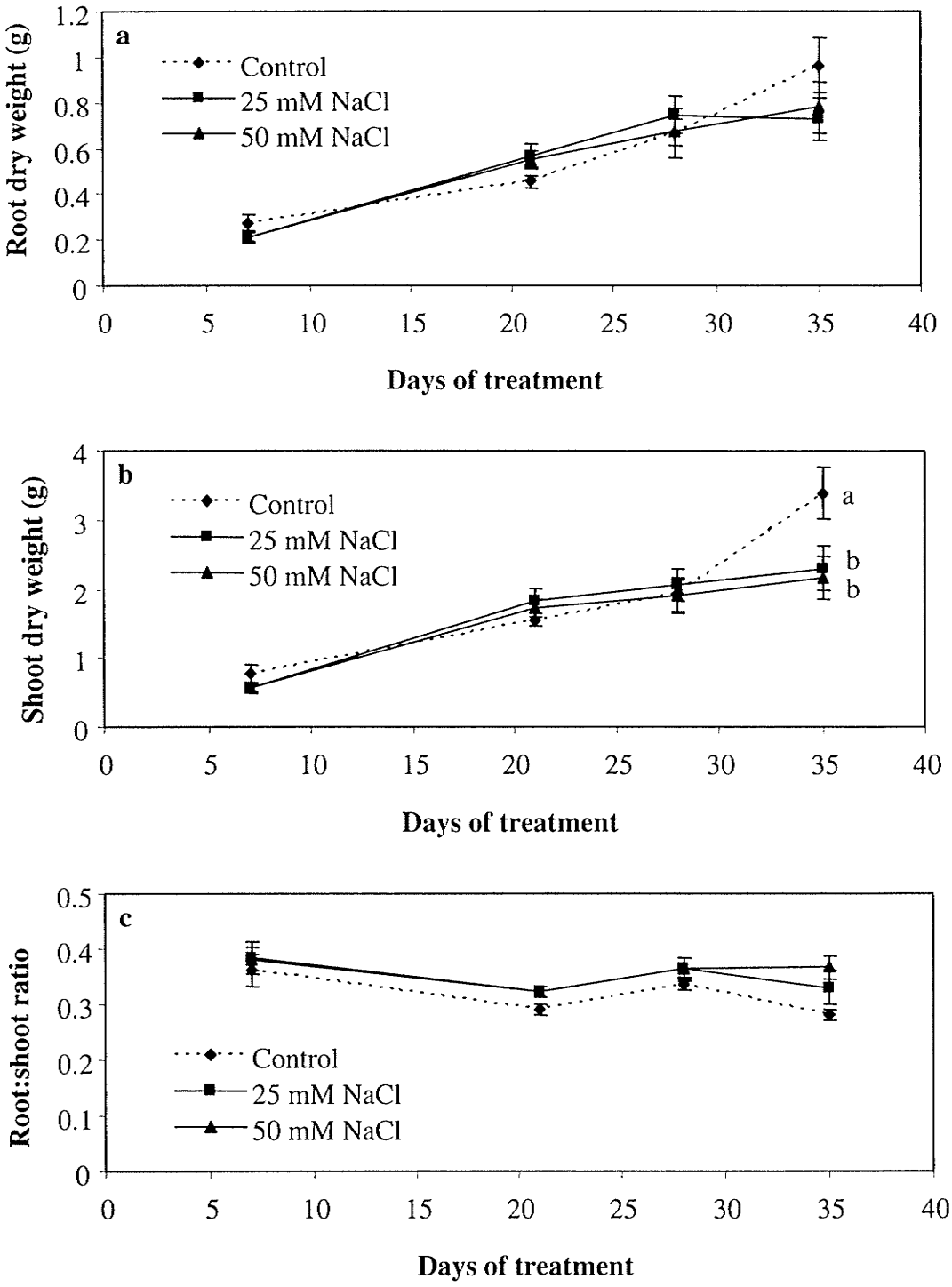

Figure 3.7 *Cornus stolonifera* seedling root dry weight (a), shoot dry weight (b), and root/shoot ratio (c), measured after 7 (n = 9), 21 (n = 18), 28 (n = 9) and 35 (n = 9) days of treatment with 0, 25 or 50 mM NaCl (mean ± SE). Letters indicate significant differences (p = 0.05) 35 days after the beginning of treatment.

3.3.2 *Photosynthesis, Pigments and Transpiration*

Transpiration rates and stomatal conductance of *C. stolonifera* leaves were significantly lower (54% and 55% respectively) in seedlings treated with 50 mM NaCl compared with the untreated seedlings (Table 3.1). Seedlings treated with 25 mM NaCl had lower transpiration rates and stomatal conductance compared to the untreated seedlings, but this difference was not significant (Table 3.1). A reduction in the amount of chlorophyll a did occur as the salt stress increased, but this difference was not significant (Table 3.2). The amount of carotenoids was not significantly reduced in seedlings treated with NaCl (Table 3.2). In addition, the ratio of chlorophyll a/ chlorophyll b was not affected by the NaCl treatment (Table 3.2). The highest salt stress (50 mM NaCl) significantly reduced the photosynthetic rate by 21.6% compared to the control seedlings (Table 3.2). The photosynthetic rate was reduced slightly (10.6%), but not significantly in seedlings treated with 25 mM NaCl compared to the untreated seedlings (Table 3.2).

3.3.3 *Soluble Carbohydrates*

The shoot tissue of *C. stolonifera* contained a greater amount of soluble carbohydrates (40.37 mg) than the root tissue (31.21 mg) after 21 days of treatment (Figure 3.8). Soluble carbohydrates from root tissue were not affected by NaCl treatment (Figure 3.8). Soluble carbohydrates increased in the shoots as the NaCl concentration increased, but the difference was not significant between the salt treated seedlings and the untreated seedlings (Figure 3.8).

3.3.4 *Water Relations and Cell Wall Elasticity*

Midday shoot water potential did not differ between NaCl treated and untreated seedlings (Table 3.3). After 7 and 21 days of treatment the shoot osmotic potential at full turgor of the control seedlings was -0.98 and -0.97 MPa respectively, and salt treatment did not significantly affect shoot osmotic potential at full turgor (Table 3.4). The shoot osmotic potential at turgor loss was lower than at full turgor, but it was also unaffected by salt treatments (Table 3.4). After seven days of treatment

Table 3.1 Leaf stomatal conductance and transpiration rate of *C. stolonifera* seedlings measured after 28 days of treatment with either 0, 25 or 50 mM NaCl (mean ± SE, n = 9). Different letters indicate significant difference (p = 0.05).

Treatment	Transpiration (mmol m^{-2} s^{-1})	Stomatal conductance (mmol m^{-2} s^{-1})
Control	3.34^{a} (± 0.17)	283.22^{a} (± 19.05)
25 mM NaCl	3.16^{a} (± 0.21)	267.78^{a} (± 17.43)
50 mM NaCl	1.53^{b} (± 0.21)	127.32^{b} (± 18.02)

Table 3.2 Pigment content and photosynthetic rate of *C. stolonifera* leaves determined at 400 µmoles photons $m^{-2} s^{-1}$ after 21 days of treatment with 0, 25 or 50 mM NaCl. (mean ± SE, n = 9). Different letters indicate significant difference (p = 0.05). FW, fresh weight.

Treatment	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Chl a and b (mg g^{-1} FW)	Chl a/b ratio	Carotenoids (mg g^{-1} FW)	Photosynthetic rate (µmol CO_2 $m^{-2} s^{-1}$)
Control	3.17 (± 0.15)	3.69 (± 0.79)	3.91 (± 0.20)	1.78 (± 0.62)	0.16 (± 0.01)	3.98^a (± 0.12)
25 mM NaCl	2.71 (± 0.24)	2.65 (± 0.57)	3.36 (± 0.31)	1.81 (± 0.56)	0.14 (± 0.01)	3.56^a (± 0.23)
50 mM NaCl	2.50 (± 0.17)	3.23 (± 0.63)	3.11 (± 0.21)	1.43 (± 0.52)	0.13 (± 0.01)	3.12^b (± 0.22)

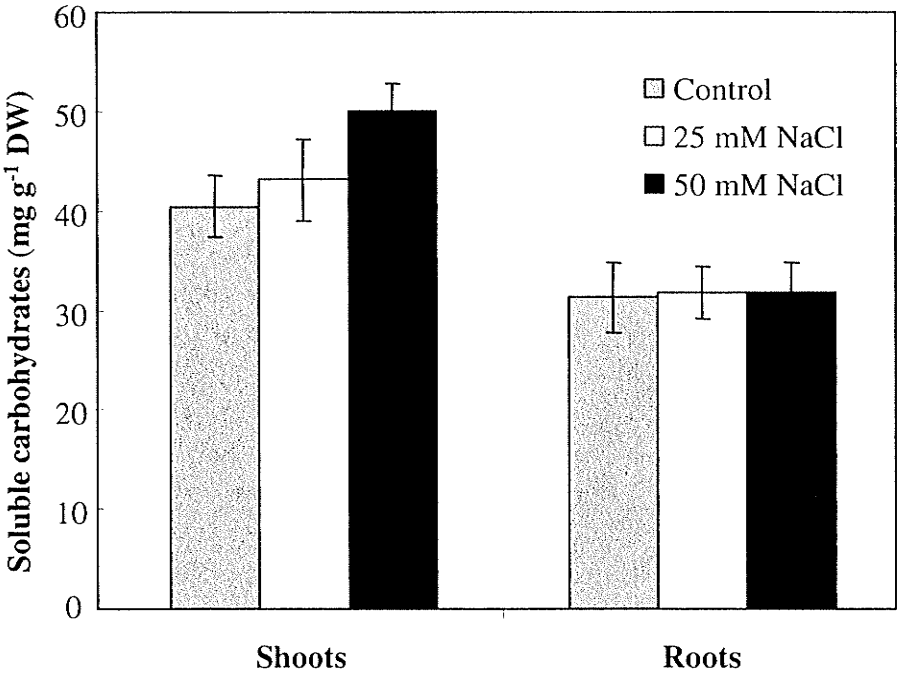

Figure 3.8 Soluble carbohydrates from *C. stolonifera* seedlings harvested 21 days after treatment with either 0, 25 or 50 mM NaCl (mean ± SE, n = 9). No significant differences were found between treatments.

Table 3.3 Shoot water potential of *C. stolonifera* seedlings measured 21 days after treatment with 0, 25 or 50 mM NaCl (mean ± SE, n = 9). No significant differences were found between treatments.

Treatment	Water Potential (MPa)
Control	-0.78 (± 0.05)
25 mM NaCl	-0.82 (± 0.05)
50 mM NaCl	-0.85 (± 0.03)

the osmotic potential at turgor loss was -1.20 MPa in the control seedlings and -1.18 MPa after 21 days of treatment. The pressure potential at full turgor was 0.67 MPa in the control seedlings after 7 days of treatment and 0.66 MPa after 21 days. There was no significant difference between treatments in the shoot pressure potential of the seedlings (Table 3.4). The relative water content at turgor loss did not differ significantly between treatments (Table 3.4). In the control seedlings the relative water content was 93.31% and 94.46% after 7 and 21 days of treatment respectively.

The control seedlings had a bulk modulus of cell wall elasticity of 8.28 MPa after 1 day of treatment. After 7 and 21 days of treatment the bulk modulus of cell wall elasticity of the control seedlings was 4.08 and 3.86 MPa respectively and did not differ between treatments (Table 3.5).

3.3.5 Cell Wall Composition

The dry shoot tissue of *C. stolonifera* contained 51% cell wall material. There was no significant difference in the amount of cell wall material extracted from shoot tissue of treated and untreated seedlings (Table 3.6). During cell wall extraction approximately 10% of the cell wall material was lost. The cell wall composition of the control plants was 24% hemicellulose, 27% pectin, 19% cellulose and 18% lignin. The hemicellulose fraction was 11.5% significantly higher in both the 25 and 50 mM NaCl treated seedlings compared with untreated seedlings (Figure 3.9). There were no significant differences between treatments in the amount of pectin, cellulose and lignin present in the cell wall of seedlings shoot tissue after 21 days of treatment (Figure 3.9). The cellulose fraction isolated from the cell wall contained 57.2%, 59.7% and 58.2% glucose for the treatments 0, 25 and 50 mM NaCl respectively. There was no difference in the amount of glucose in the cellulose fraction between treatments.

Table 3.4 Water relation parameters of *C. stolonifera* shoots calculated from P-V curves. $\Psi_{\pi 100}$, osmotic potential at full turgor; $\psi_{\pi 0}$, osmotic potential at turgor loss; ψ_{P100}, pressure potential at full turgor; RWC_0, relative water content at turgor loss, (mean ± SE, n = 8). No significant differences were found between treatments.

Days of Treatment	Treatment	$\psi_{\pi 100}$ (MPa)	$\psi_{\pi 0}$ (MPa)	ψ_{P100} (MPa)	RWC_0 (%)
7	Control	-0.98 (± 0.05)	-1.20 (± 0.08)	0.67 (± 0.06)	93.31 (± 0.92)
7	25 mM NaCl	-0.98 (± 0.05)	-1.19 (± 0.05)	0.65 (± 0.05)	94.03 (± 0.71)
7	50 mM NaCl	-0.90 (± 0.04)	-1.11 (± 0.05)	0.61 (± 0.03)	95.31 (± 0.58)
21	Control	-0.97 (± 0.05)	-1.18 (± 0.08)	0.66 (± 0.09)	94.46 (± 0.83)
21	25 mM NaCl	-0.95 (± 0.05)	-1.15 (± 0.07)	0.58 (± 0.06)	96.46 (± 0.45)
21	50 mM NaCl	-0.97 (± 0.07)	-1.19 (± 0.10)	0.65 (± 0.07)	96.32 (± 0.56)

Table 3.5 Bulk modulus of cell wall elasticity (ε) of *C. stolonifera* seedling shoot tissue measured after 1 (n = 15), 7 (n = 8) and 21 (n = 8) days of treatment with 0, 25 or 50 mM NaCl (mean ± SE). No significant differences were found between treatments.

Days of treatment	Control ε (MPa)	25 mM NaCl ε (MPa)	50 mM NaCl ε (MPa)
1[a]	8.28 (± 0.85)	8.37 (± 0.67)	8.80 (± 1.19)
7	4.08 (± 0.55)	3.91 (± 0.47)	3.29 (± 0.27)
21	3.86 (± 0.38)	3.91 (± 0.70)	3.73 (± 0.30)

[a] ε values for 1 day of treatment were determined from a preliminary experiment.

Table 3.6 Percentage of cell wall extracted from shoot tissue of *C. stolonifera* seedlings after 21 days of treatment with 0, 25 or 50 mM NaCl (mean ± SE, n = 9). No significant differences were found between treatments.

	Cell wall (% of shoot)
Control	50.94 (± 1.85)
25 mM NaCl	50.19 (± 1.43)
50 mM NaCl	49.04 (± 1.15)

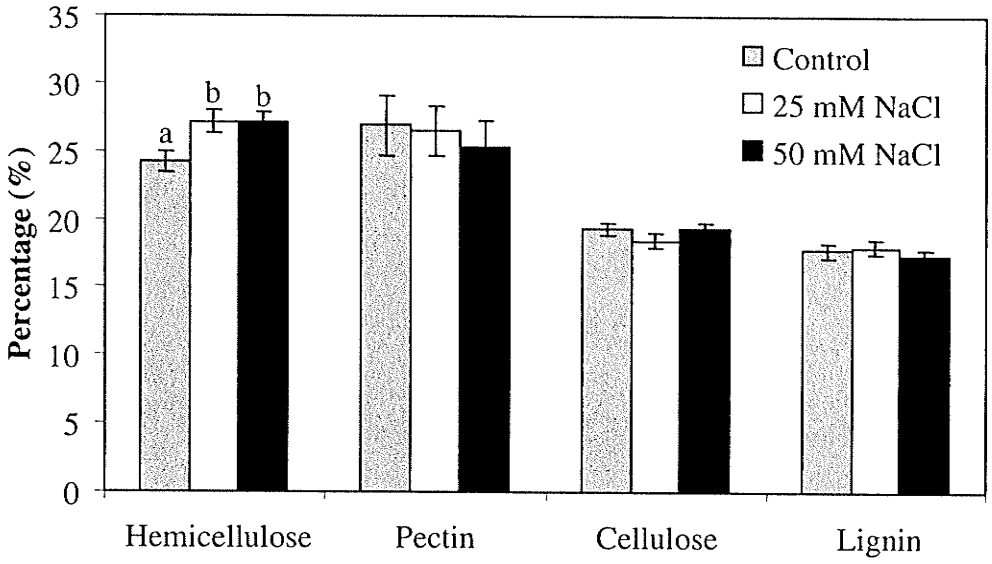

Figure 3.9 Cell wall composition of *C. stolonifera* shoot tissue after 21 days of treatment with 0, 25 or 50 mM NaCl (mean ± SE, n = 9). Values presented are expressed as a percentage of the total cell wall material extracted. Percentage of cellulose presented was determined from the amount of glucose present in the cellulose fraction. The percent of cell wall lost during separation of fractions was approximately 10%. Letters indicate significant differences (p = 0.05).

The most predominant carbohydrate in the pectin fraction of the untreated seedlings was arabinose (35.4%) (Table 3.7). Other carbohydrates identified in the pectin fraction of untreated seedlings included galacturonic acid (24.2%), glucose (14.3%), galactose (13.95%), mannose (9.2%) and rhamnose (3.0%). No significant differences were found in the composition of the pectin fraction between the treated and untreated seedlings (Table 3.7). Xylose comprised 55.6% of the hemicellulose fraction in the control seedlings. Other carbohydrates present in the hemicellulose fraction of the control seedlings were glucose (27.2%), galactose (9.6%), arabinose (7.3%), and rhamnose (0.3%). Fucose and mannose were also found in the hemicellulose fraction at very low levels (less than 0.5%) in the treated seedlings but not in the untreated seedlings (Table 3.8). No significant differences were found in the composition of the hemicellulose fraction between the treated and the untreated seedlings (Table 3.8).

Table 3.7 Composition of the pectin fraction extracted from shoot cell walls of *C. stolonifera* seedlings, after 21 days of treatment (mean ± SE, n = 6). Values expressed are a percentage of all sugars identified in the pectin fraction. No significant differences were found between treatments.

	Control (%)	**25 mM NaCl** (%)	**50 mM NaCl** (%)
Rhamnose	3.0 (± 0.8)	5.8 (± 0.56)	6.1 (± 0.4)
Arabinose	35.4 (± 1.1)	36.2 (± 1.6)	34.6 (± 0.9)
Galacturonic Acid	24.2 (±1.3)	23.3 (± 1.3)	18.1 (± 1.4)
Mannose	9.2 (± 1.0)	3.4 (± 1.4)	2.6 (± 1.6)
Galactose	13.9 (± 0.9)	16.2 (± 1.0)	18.0 (± 1.7)
Glucose	14.3 (± 1.6)	15.1 (± 2.8)	20.6 (± 2.5)

Table 3.8 Composition of the hemicellulose fraction of cell walls of *C. stolonifera* seedlings, after 21 days of treatment (mean ± SE, n = 6). Values expressed are a percentage of all sugars identified in the hemicellulose fraction. No significant differences were found between treatments.

	Control (%)	**25 mM NaCl** (%)	**50 mM NaCl** (%)
Arabinose	7.3 (± 0.3)	7.3 (± 0.6)	8.0 (± 0.7)
Xylose	55.6 (± 2.9)	53.9 (± 5.3)	49.6 (± 2.1)
Galactose	9.6 (± 0.9)	10.5 (± 0.7)	10.6 (± 0.6)
Glucose	27.2 (± 2.1)	28.0 (± 4.8)	31.0 (± 2.4)
Rhamnose	0.3 (± 0.3)	0.3 (± 0.3)	0.3 (± 0.3)
Fucose	0	0	0.3 (± 0.3)
Mannose	0	0	0.2 (± 0.3)

3.4 Discussion

3.4.1 Growth

Salt stress reduces growth in a number of woody plant species. Shoot dry weight of sweet orange seedlings (*Citrus sinensis*) was reduced at concentrations above 45 mM NaCl and root dry weight was reduced at concentrations of 60 mM NaCl (Bañuls and Primo-Millo 1992). In poplar (*Populus deltoides*) 85 mM NaCl (Fung et al. 1998), and Acacia (*Acacia nilotica*), 100 mM NaCl (Nabil and Coudret 1995), reduced shoot dry weight and height growth. In this study, 50 mM NaCl caused a reduction of the shoot dry weight of *C. stolonifera* seedlings while shoot height and root dry weight were unaffected; however as the effect of treatment varied with time, greater decreases in growth may become apparent if the duration of salt stress increased. Previous work with *C. stolonifera* has shown that a 100 mM NaCl concentration was necessary to reduce shoot height (Renault et al. 2001b). A reduction in both shoot dry weight and height were found in *C. stolonifera* seedlings treated with saline composite tailings (CT) water although the NaCl concentration was less than 50 mM (Renault et al. 2001a). These tailings contained a number of detrimental ions (boron, aluminum and strontium) and hydrocarbons that may have affected growth in addition to the effects of NaCl (Renault et al. 2001a).

Root growth is often less affected than shoot growth under salt stress, resulting in larger root/shoot ratios (Munns and Termat 1986; Shannon et al. 1994). In this study the root/shoot ratio was slightly higher in treated plants, but not significantly different from the control. An increase in the root/shoot ratio indicates a reduction in the leaf area while root growth is maintained, allowing for continued uptake of nutrients and water (Shannon et al. 1994). The specific mechanism that regulates reduced shoot growth during salt stress is not fully understood and a number of processes may contribute (Fung et al. 1998). As the effect of salt stress on growth is very rapid and is quickly reversed when the stress is removed, decreased growth has been attributed primarily to osmotic stress (Munns and Termat 1986; Neumann 1993).

Ion toxicity may also contribute to decreased growth, particularly in perennial species that are exposed to the salt stress for a long period of time; ions accumulate in plant tissue decreasing cellular metabolism (Munns and Termat 1986). Previous work with *C. stolonifera* has shown that both Na^+ and Cl^- accumulate in root and shoot tissue under NaCl stress (Renault et al. 2001b). The ion accumulation may contribute to decreased growth due to ion toxicity. Salinity altered nutrient uptake in Italian ryegrass (*Lolium multiflorum*) as NaCl caused nitrogen deficiency (Veen and Kleinendorst 1985). Treatment of *C. stolonifera* with 50 mM NaCl reduced the concentration of K^+ in root tissue, and Mg^{2+} in shoot tissue (Renault et al. 2001b). Decreases in K^+ and Mg^{2+} may lead to nutrient deficiencies, further decreasing growth. During salt stress a reduction in cell wall expansion due to cell wall hardening has been found in tobacco (*Nicotiana tabacum*) (Iraki et al. 1989a), chick-pea (*Cicer arietinum*) (Muñoz et al. 1993) and maize (*Zea mays*) (Neumann 1993; Neumann 1994) and this may also contribute to decreased growth.

3.4.2 Photosynthesis, Pigments and Transpiration

The reductions in photosynthesis recorded in this study may also contribute to decreased growth and have been observed in a number of salt stressed plants (Bañuls and Primo-Millo 1992; Wang et al. 1997; Fung et al. 1998). In poplar (*Populus deltoides*) treated with high salt concentrations (170 mM NaCl), photosynthetic rates were reduced much more rapidly than when seedlings were treated with lower concentrations (85 mM NaCl) (Fung et al. 1998). Photosynthetic rates are thought to be reduced under salt stress for a number of reasons including inhibition or altering of photosynthetic enzyme activity, changes in the structure and function of photosynthetic organelles, decreased CO_2 availability due to increased stomatal resistance and/or inhibition of the light reactions (Hagemeyer 1997; Reddy et al. 1997). Although decreased photosynthetic rates may contribute to decreased growth, not all findings support this (Munns 1993). Sodium chloride stressed spinach (*Spinacia oleracea*) did not show a decrease in the photosynthetic potential of the leaves even though growth was significantly reduced (Robinson et al. 1983). It has been suggested that photosynthetic rates may be reduced due to an accumulation of

sugars within the mesophyll cells causing a feedback inhibition of photosynthesis (Herold 1980; Munns 1993).

Despite reductions in photosynthetic rates, NaCl stress did not affect the amount of photosynthetic pigments in *C. stolonifera* seedlings; however the reduction of chlorophyll a as salt concentration increased may contribute to decreased photosynthesis. These findings support previous work done with *C. stolonifera* that did not find a reduction in chlorophyll a or chlorophyll b when treated with NaCl (Renault et al. 2001b). The absence of a large decrease in the amount of photosynthetic pigments indicates reductions in photosynthesis may be due to a decrease in the CO_2 availability as salt stress reduced the transpiration rate and stomatal conductance in *C. stolonifera* seedlings treated with 50 mM NaCl both in this experiment and in previous work (Renault et al. 2001b).

Increased stomatal resistance may facilitate water conservation in *C. stolonifera* as less water would be lost through transpiration. However, increased stomatal resistance is not an efficient long-term salt tolerance mechanism as a reduction in gas exchange would negatively impact photosynthetic rates and growth (Hasegawa et al. 2000). Work done with CT and *C. stolonifera* suggest that although transpiration is reduced under salt stress, water uptake is still relatively high and that there must be other mechanisms to restrict the uptake and accumulation of Cl^- and Na^+ to prevent ionic toxicity (Renault et al. 1998). Subsequent work has found that *C. stolonifera* restricts the movement of Na^+ and Cl^- from the roots into the shoots (Renault et al. 2001a). Increased stomatal resistance under salt stress has been observed in a number of species including sweet orange (*Citrus sinensis*) (Bañuls and Primo-Millo 1992) and poplar (*Populus deltoides*) (Fung et al. 1998). In bean (*Phaseolus vulgaris)* salt stress decreased the number of stomata, improving water conservation through decreased transpiration. The morphology of *C. stolonifera* leaves was not examined during this study, but it is recommended for future work to determine if stomatal resistance is increased due to stomatal closure or changes in stomtatal density.

3.4.3 Water Relations and Cell Wall Elasticity

Despite the effects of NaCl stress on photosynthesis and stomatal resistance, it did not affect the shoot water potential of *C. stolonifera*. Not only was the shoot water potential of *C. stolonifera* not reduced by salinity but neither the pressure potential nor the osmotic potential (both at full turgor and turgor loss) were affected. These findings indicate that the stress imposed on actively growing *C. stolonifera* seedlings with 50 mM NaCl had little effect on shoot water relations. These findings differ from what has been reported for a number of species; however, the concentration of salt used was higher than the one used during this study. Poplar (*Populus deltoides*) treated with 75 mM NaCl had a significantly reduced leaf water potential compared to untreated plants (Fung et al. 1998). Leaf osmotic potential at full turgor and turgor loss and water potential were reduced in olive trees (*Olea europaea*) (Gucci and Tattini 1997) treated with 200 mM NaCl. In Acacia (*Acacia nilotica*), 100 mM NaCl reduced both leaf water and leaf osmotic potential (Nabil and Coudret 1995).

Previous work with *C. stolonifera* has yielded variable results of the effect of salt stress on shoot water potential. Treatment with saline CT water did not affect the shoot water potential of *C. stolonifera*, but there was a decrease in the shoot water potential when CT water was supplemented with 3 g L^{-1} Na_2SO_4 (Renault et al. 1998). Further work with CT water showed an increase in the shoot water potential after one day of treatment, a decrease one week later and then no effect after four weeks of salt stress (Renault et al. 1999). Subsequent work with CT water showed that salt stress increased the shoot water potential in *C. stolonifera* seedlings four weeks after treatment (Renault et al. 2001a). In addition, treatment with 100 mM NaCl and 50 mM Na_2SO_4, but not with lower concentrations, decreased the shoot water potential after four weeks of treatment (Renault et al. 2001b). Hummel et al. (1979) examined the variability of leaf water potential of *C. stolonifera* and found significant variation among plants grown in the field, but not for plants grown in a growth chamber. As conditions during this experiment were more similar to the conditions in a growth chamber than field conditions, it can be assumed that the absence of significant differences between treatments is valid and is not because variability in shoot water

potential was high. From the results of this study and previous work, the shoot water potential of *C. stolonifera* does not appear to be affected below a certain threshold of salt stress; however, once that threshold is reached the water potential is reduced. The osmotic potentials of the nutrient solutions in this experiment were higher than the water potentials of the plant tissues, but the gradients between the plant and water potential of the rooting medium decreased as the salt concentration increased, suggesting that water absorption was more difficult as the salt concentration increased.

The osmotic potential at full turgor for unstressed *C. stolonifera* seedlings was between -0.97 and -0.98 MPa. At turgor loss the osmotic potential was -1.19 MPa. These values of osmotic potential are higher than those reported for sweet orange (*Citrus sinensis*) (-2.28 MPa) (Bañuls and Primo-Millo 1992) and white spruce (*Picea glauca*) (-2.93 MPa) (Wang and Zwiazek 1999); however, they are similar to osmotic potentials reported for flowering dogwood (*Cornus florida)* (-0.31 to -1.34 MPa), red maple (*Acer rubrum*) (-0.58 to -1.51 MPa) (Gebre et al. 1998), and gray birch (*Betula populifolia*) (-0.92 MPa) (Morse et al. 1993). Neither the osmotic potential of *C. stolonifera* seedlings nor the amount of soluble carbohydrates were affected by the salt stress. This finding is similar to water stressed white spruce (*Picea glauca*) for which the osmotic potential at full turgor was not reduced by the stress (Koppenaal et al. 1991). A reduction in osmotic potential occurs in a number of species, allowing for continued water uptake (Bañuls and Primo-Millo 1992; Nabil and Coudret 1995; Clifford et al. 1998). Flowering dogwood (*Cornus florida*) adjusted osmotically during periods of low soil water potential (Gebre et al. 1998; Tschaplinski et al. 1998), indicating that osmotic adjustment is used within the *Cornus* genus as a stress tolerance mechanism. The absence of both a decrease in osmotic potential and accumulation of soluble carbohydrates may indicate that osmotic adjustment does not contribute to salt stress tolerance in *C. stolonifera*; however it may also indicate that there is little need for adjustment as the water stress was minimal. These findings are preliminary, and more work will be needed to fully understand osmotic adjustment in

C. stolonifera under NaCl stress, as adjustment with solutes other than carbohydrates may be occurring within the roots.

The average pressure potential of *C. stolonifera* shoot tissue was 0.67 MPa. The pressure potential found for *C. stolonifera* is much lower than pressure potential values reported for other woody species. White spruce (*Picea glauca*) seedlings had a pressure potential of 1.71 MPa (Marshall and Dumbroff 1999) and red maple (*Acer rubrum*) had a pressure potential of 1.5 MPa (Roberts et al. 1981). Values for *C. stolonifera* were more similar to values determined for sweet orange (*Citrus sinensis*) which had a pressure potential of 1.07 MPa (Bañuls and Primo-Millo 1992). The large variation in pressure potential values reported for woody species may be due to species differences or the age of the tissue used for the measurements, as the pressure potential of old American holly (*Ilex opaca*) leaves was 1.59 MPa while the pressure potential of new leaves was 0.84 MPa (Roberts et al. 1981). Salt treatment did not alter the pressure potential of *C. stolonifera* seedlings. The absence of an effect of salt stress on the pressure potential was expected as no change was found in water potential or osmotic potential. In sweet orange (*Citrus sinensis*) the pressure potential was not affected by salt stress (Bañuls and Primo-Millo 1992); however in white spruce (*Picea glauca*) (Marshall and Dumbroff 1999) and loblolly pine (*Pinus taeda*) (Meier et al. 1992), drought stress significantly reduced the pressure potential. The effects of salt stress on the shoot pressure potential is not fully understood and depends on a number of factors, particularly cell wall elasticity and relative water content.

The relative water content at turgor loss of *C. stolonifera* shoots was between 93% and 94% of the total water volume. These results were similar to those reported for black spruce (*Picea mariana*), which had a relative water content at turgor loss of 94% (Redfield 2001); however they were much higher than those reported for other woody species. In white spruce (*Picea glauca*), the water potential at turgor loss was between 76% and 85%, varying with the time of year (Wang and Zwiazek 1999). The relative water content at turgor loss was approximately 87% in lemon (*Citrus limon*), (Ruiz-Sanchez et al. 1997) and 87.5% in black spruce (*Picea mariana*) (Blake et al.

1991). The relative water content at turgor loss in shoot tissue was not affected by NaCl stress in *C. stolonifera* seedlings. In white spruce (*Picea glauca*) needles, the relative water content at turgor loss was reduced after drought stress (Marshall and Dumbroff 1999). A reduction in the relative water content at turgor loss indicates that changes, such as increased cell wall elasticity, must be occurring. In the case of white spruce (*Picea glauca*), cell wall elasticity increased, allowing for turgor maintenance at lower water volumes (Marshall and Dumbroff 1999). In lemon (*Citrus limon*), drought stress reduced the relative water content at turgor loss in mature leaves after two periods of drought stress and was also accompanied by an increase in cell wall elasticity (Ruiz-Sánchez et al. 1997). In olive (*Olea europaea*) seedlings treated with NaCl, the relative water content at turgor loss decreased and was found to contribute to salt tolerance although cell wall elasticity was not examined in this species (Gucci and Tattini 1997).

The bulk modulus of cell wall elasticity determined for *C. stolonifera* (3.9-8.3 MPa) was similar to values found for gray birch (*Betula populifolia*) (4.9-6.8) (Morse et al. 1993), ber (*Ziziphus mauritiana*) (6.2 MPa) (Clifford et al. 1998), and jack pine (*Pinus banksiana*) (5.1 MPa) (Fan et al. 1994), but was lower than values determined for flowering dogwood (*Cornus florida)* (15.0 MPa) (Roberts et al. 1981), white spruce (*Picea glauca*) (14.5 MPa) (Marshall and Dumbroff 1999), mangrove (*Avicennia germinans*) (19.4 MPa) (Suarez and Sobrado 2000), and red maple (*Acer rubrum*) (17.0 MPa) (Roberts et al. 1981). The tissue, age, and growing conditions of the plants used to determine cell wall elasticity varied between species and these factors could have contributed to the differences found. The low bulk modulus of cell wall elasticity for *C. stolonifera* indicates the cell walls in shoot tissue are elastic, which is also supported by the relatively low pressure potential of *C. stolonifera*; however the high relative water content at turgor loss does not indicate the presence of elastic cell walls which would be able to shrink around the protoplast maintaining turgor pressure as the water volume decreased.

Although salt stress did not affect shoot cell wall elasticity in *C. stolonifera* seedlings, salt and osmotic stresses have been found to alter cell wall elasticity,

contributing to increased stress tolerance in a number of other species. In Douglas-fir (*Pseudotsuga menziesii*), drought stress increased cell wall elasticity helping to regulate turgor pressure during stress in the absence of osmotic adjustment (Joly and Zaerr 1987). In mangrove seedlings (*Aviccennia germinans*) treated with NaCl, both cell wall elasticity and solute concentration increased, maintaining water uptake and turgor pressure at low soil water potentials (Suárez et al. 1998). Increased cell wall elasticity contributed to stress tolerance in lemon (*Citrus limon*) (Ruiz-Sanchez et al. 1997) and black spruce (*Picea mariana*) (Blake et al. 1991) under drought stress. In contrast increased cell wall rigidity in combination with osmotic adjustment allowed a positive water balance to be maintained during NaCl stress in the relatively salt tolerant species Acacia (A*cacia nilotica*) (Nabil and Coudret 1995), alligator weed (*Alternanthera philoxeroides*) (Bolaños and Longstreth 1984), and mangrove seedlings (*Avicennia germinans*) (Suarez and Sobrado 2000). A similar type of adjustment was observed in ber (*Ziziphus mauritiana*) during drought stress as more rigid cell walls and osmotic adjustment increased drought tolerance (Clifford et al. 1998). Although neither an increase nor decrease in cell wall elasticity occurred in the shoot tissue of *C. stolonifera*, adjustments to cell wall elasticity may occur in the root tissue, as was found in jack pine. In jack pine (*Pinus banksiana*), alterations to the cell wall of root cells permitted the cell walls to constrict around the protoplasts and maintain turgor as water volume decreased (Marshall et al. 1999). Further work will be required to study how salt stress affects root water relations and cell wall elasticity of *C. stolonifera* seedlings.

3.4.4 Cell Wall Composition

Salt and drought stress can affect cell wall composition, but the results are variable depending on the species and level of stress. In this study NaCl stress did not alter the amount of cell wall material in shoot tissue of *C. stolonifera*, while cell wall material increased in chick-pea (*Cicer arietinum*) epicotyls during osmotic stress (Muñoz et al. 1993). Treatment with 170 mM NaCl reduced cell wall material in English scurvy grass (*Cochlearia anglica*) (Binet 1985), whereas NaCl did not affect the amount of cell wall material in bean (*Phaseolus vulgaris*) (Binet 1985). Salt stress

did not affect cell wall structural properties of maize leaves (*Zea mays*) after short-term salinity (Cramer et al. 2001).

The cell walls of untreated *C. stolonifera* seedlings contained 24.2% hemicellulose; this finding is similar to other woody plants which can contain up to 30% hemicellulose in their cell walls (Bikova and Treimanis 2002). White spruce (*Picea glauca*) contained 32% hemicellulose in the cell walls of needles (Zwiazek 1991). Salt stress increased the amount of hemicellulose in *C. stolonifera* seedlings and is supported by other findings. Zwiazek (1991) found an increase in the hemicellulose fraction of the cell wall in white spruce (*Picea glauca*) needles after repeated drought stress. Hemicellulose accounted for 41% of cell wall material in tobacco (*Nicotiana tobacum)* adapted to NaCl compared with 30% in unadapted cells (Iraki et al 1989a). An increase in the hemicellulose content in water stressed wheat (*Triticum aestivum*) coleoptiles after 3 days of treatment was observed (Wakabayashi et al. 1997).

An increase in the amount of hemicellulose in the cell wall may play a role in stress tolerance (Zwiazek 1991). Hemicellulose functions to form a network linking cellulose fibers together. An increase in hemicellulose may make this network more rigid, decreasing the ability of the cell wall to expand and contributing to decreased growth. Research on rice (*Orzya sativa*) suggested that salt stress caused an increase in the activity of peroxidase enzymes that function in the formation of cross-links between cell wall components and that this increase was associated with decreased root growth (Lin and Kao 1999).

The untreated *C. stolonifera* seedlings had a similar hemicellulose composition to that reported in the literature. In chick-pea (*Cicer arietinum*) epicotyls xylose and glucose were the primary sugars in the hemicellulose, (Muñoz et al. 1993). This was the similar to *C. stolonifera* as xylose was the main sugar followed by glucose. In white spruce (*Picea glauca*), the hemicellulose composition differed as galacturonic acid was the primary sugar and xylose was present but only accounted for 17.6% of the hemicellulose fraction (Zwiazek 1991). In *C. stolonifera,* xylose accounted for 55.6% of the hemicellulose fraction of the cell wall. Differences

between species regarding hemicellulose composition may be attributed to a number of factors, including species differences, developmental stage and/or the plant organ(s) examined since only the hemicellulose composition from needles was examined in white spruce (*Picea glauca*) (Zwiazek 1991), while the entire shoot tissue was examined for *C. stolonifera*. It has also been suggested that the direct comparison of hemicellulose values from different plants is unsuitable as variation in the method of extraction will be introduced by the researchers (Wilkie 1985). The composition of the hemicellulose fraction was not affected by NaCl stress in *C. stolonifera*. This was similar to white spruce (*Picea glauca*), as drought stress did not alter hemicellulose composition (Zwiazek 1991).

The amount of pectin in the cell wall of *C. stolonifera* was similar to that reported for white spruce needles (*Picea glauca*); however in spruce (*Picea glauca*) uronic acids (galacturonic and glucuronic acid) (Zwiazek 1991) were the predominant sugars, while arabinose was the major sugar in the pectin fraction of *C. stolonifera* followed by galacturonic acid. Rhamnose, mannose, galactose and glucose were present in the pectin fraction of white spruce (*Picea glauca*) (Zwiazek 1991) in very similar amounts to those found in the pectin fraction of *C. stolonifera* cell walls. These findings differ from chick-pea (*Cicer arietinum*) epicotyls, which contained primarily galactose and arabinose in the pectin fraction while the presence of uronic acids was not reported (Muñoz et al. 1993). The reasons for the variability observed in pectin composition of different species are not fully understood. It may partially be due to the method of extraction. Zwiazek (1991) silylated the samples, while the samples were acetylated during this study.

Sodium chloride stress did not affect the amount or composition of pectin in *C. stolonifera* seedlings during this study. Drought stress did not alter the pectin fraction in chick-pea epicotyls (*Cicer arietinum*) (Muñoz 1993); however, a change in the pectin fraction occurred in a number of species during stress. Drought stress decreased the amount of pectin present in the cell wall of white spruce seedlings (*Picea glauca*) (Zwiazek 1991). Tobacco (*Nicotiana tobacum)* cells unadapted to NaCl had a much larger fraction of pectin extracted with ammonium oxalate

compared to NaCl adapted cells, suggesting that the pectin fraction was more loosely bound in salt adapted cells (Iraki et al 1989a, 1989b). In sea star (*Aster tripolium*), the pectin fraction contained a higher amount of arabinose and a lower amount of glucose in the cell wall of salt stressed plants compared to unstressed plants (Binet 1985); as sea star (*Aster tripolium*) is a halophyte it is assumed that these changes in the pectin fraction would increase salt tolerance. In white spruce (*Picea glauca*), drought stress increased the amount of arabinose and mannose and decreased the amount of galactose and glucose in the pectin fraction (Zwiazek 1991). Changes in the amount of arabinose and galactose in the pectin fraction have been implicated in cell wall loosening and extensibility (Muñoz et al. 1993). Changes in the proportions of sugars in the pectin fraction may affect cell wall strength and cell wall elasticity (Zwiazek 1991), but it is not yet fully understood why changes in the pectin fraction occur during stress.

The cell walls in shoot tissue of unstressed *C. stolonifera* contained 19.3% cellulose, which falls within the wide range reported for the amount of cellulose in plant cell walls. Krishnamurthy (1999) reported that primary cell walls contain 1 to 10% cellulose and secondary cell walls can have much as 50% cellulose. The shoot tissue examined for *C. stolonifera* contained cells with both primary and secondary cell walls; therefore it is difficult to compare these values directly. In white spruce needles (*Picea glauca*), 13% of the cell wall was cellulose (Zwiazek 1991), which is very similar to *C. stolonifera*. The amount of glucose present in the cellulose fraction extracted from *C. stolonifera* was used to determine that only 57.2 to 59.7% of the fraction was actually cellulose. These results were expected as the cellulose fraction is the final fraction extracted from the cell wall and often contains other sugars such as mannose, xylose and galacturonic acid that were not fully removed with the pectin fraction and hemicelluloses, such as glucomannan and xyloglucan, may have also be present in this fraction (Brett and Waldron 1990). There may also have been some lignin that was not fully removed.

Salt and osmotic stress decreased the amount of cellulose in the cell walls of a number of plant species including wheat (*Triticum aestivum*) (Wakabayashi et al.

1997), cotton (*Gossypium hirsutum*) (Zhong and Lauchli 1998), and tobacco (*Nicotiana tobacum*) (Iraki et al. 1989a,1989b), but results are variable. Salt stress did not affect the cellulose content in cell walls of *C. stolonifera* shoot tissue. The cellulose content of the cell wall was also not affected in white spruce (*Picea glauca*) seedlings during drought stress (Zwiazek 1991). The increase in the cellulose content in the cell wall of pea (*Pisum sativum*) roots when exposed to 120 mM NaCl was attributed to increased cellulose synthesis (Solomon et al. 1987). Decreased cellulose in the cell wall has been attributed to a decrease in cellulose synthesis (Zhong and Lauchli 1988; Iraki et al. 1989b). Cellulose synthesis occurs at the outer plasma membrane surface and it is catalyzed by a number of cellulose synthase enzymes which are very sensitive to membrane disruption (Buchanan et al. 2000). In cotton (*Gossypium hirustum*), salt stress imposed by 150 mM NaCl reduced cellulose synthesis probably due to a disruption of the plasma membrane as Na^+ replaced Ca^{2+} (Zhong and Lauchli 1998). The absence of an effect of NaCl on the amount of cellulose in the cell wall of *C. stolonifera* may indicate the ability of the plant to restrict the accumulation of ions in shoot tissues (Renault et al. 2001a), reducing or preventing membrane disruption.

Lignin accounted for 17.7% of cell wall material in *C. stolonifera*, which is very similar to the amount of lignin reported in cell walls of white spruce needles (*Picea glauca*) (Zwiazek 1991). Similar to findings for *C. stolonifera*, cell wall lignification was not affected by salt stress in tomato (*Lycopersicon esculentum*) roots (Peyrano et al. 1997) or by water stress in alfalfa (*Medicago sativa*) (Deetz et al. 1996). However, changes in cell wall lignification have been found in a number of species during stress. A reduction in the lignification of rice (*Oryza sativa*) (Lin and Kao 2001) and Chenopodiaceae (*Atriplex prostrate*) (Wang et al. 1997) occurred during salt stress. In white spruce needles (*Picea glauca*), drought stress caused a decrease in cell wall lignification (Zwiazek 1991). In pea (*Pisum sativum*) root cells exposed to 120 mM NaCl, the lignin content of the cell wall increased (Solomon et al. 1987). Changes in cell wall lignin content affects the cell wall physical properties such as cell wall elasticity (Zwiazek 1991) and the presence of lignin in the secondary

cell walls of non-growing cells increases the strength and rigidity of the cell walls (Raven et al. 1981), suggesting increased lignification during salt stress may increase cell wall rigidity and result in a decreased ability for cell wall expansion.

3.5 Summary

Treatment of actively growing *C. stolonifera* seedlings with 50 mM NaCl reduced shoot dry weight after 35 days of treatment. The reduction in shoot growth was probably a consequence of lower photosynthetic rates. A number of factors, such as changes in photosynthetic enzyme activity and reductions in chlorophyll a, may have contributed to the decrease in photosynthesis, but increased stomatal resistance was likely the main contributing factor. The water potential, cell wall elasticity, pressure potential and the solute potential of the shoot tissue were unaffected by the salt stress suggesting little effect of the stress. However, the water relation parameters were determined before the effect on growth was observed and a longer term study is recommended. The only change in cell wall composition was an increase in the hemicellulose fraction of both the 25 mM and 50 mM NaCl treated plants. This increase in hemicellulose may contribute to salt tolerance, but it is not fully understood.

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4.0 The Effect of NaCl on Cold Acclimated *Cornus stolonifera* Seedlings during Bud Break

4.1 Introduction

Salt stress is very detrimental to plants as it causes both ion toxicity due to the accumulation of ions within plant tissue, and osmotic stress due to decreased soil water potential. Plants vary in the level of their salt tolerance. Halophytes are extremely salt tolerant whereas glycophytes are much less salt tolerant, with some variations depending on the species. Plant age, stage of development, and life history influence stress tolerance (Greenway and Munns 1980). Events during the life history of a plant such as stress preconditioning can affect stress tolerance. Water stress preconditioning in poplar (*Populus deltoides*) increased tolerance when preconditioned seedlings were exposed to subsequent drought stress (Gebre and Kuhns 1993). Plant age and stage of development have also been associated with different levels of stress tolerance. The seeds of woody plants are generally tolerant of saline conditions while, during germination and as young seedlings woody plants are very sensitive to salt stress (Shannon et al. 1994). The salt stress tolerance of *C. stolonifera* differs between germinating seeds, 3 month and 6 month old seedlings (Renault, personal communication; Renault et al. 2000; Renault et al. 2001b). Growth was reduced more in 3 month old *C. stolonifera* seedlings compared to 6 month old seedlings when treated with saline fine tailings, thus suggesting that the stage of development of *C. stolonifera* plays an important role in stress tolerance (Renault et al. 2000).

The process of cold acclimation, which many woody plants undergo in the fall to reach a dormant state, causes metabolic and physiological changes that may affect subsequent stress tolerance (Lang 1996). Previous work has shown that *C. stolonifera* undergoes a number of changes during cold acclimation such as a reduction in water content, changes in transpiration rates (McKenzie et al. 1974; Parsons 1978), an increase in the amount of soluble carbohydrates (Ashworth et al. 1993), and

accumulation of a protein related to dehydrins (Sarnighausen et al. 2002). During dormancy plants are considered to be very stress tolerant, while during bud break stress tolerance is thought to decrease (Lang 1996). Understanding how woody plants tolerate salt stress during bud break is important as dormant seedlings are often used for reforestation and may be planted on saline sites, such as the oil sands tailings in northeastern Alberta. It has been suggested that as the germination stage is strongly affected by salinity, older, more tolerant seedlings should be planted to reclaim mine tailing sites as establishment and survival rates would be higher (Redfield and Zwiazek 2000).

This study examined the effects of NaCl stress on *C. stolonifera* seedlings during bud break. Water relation parameters including shoot osmotic potential at full turgor, shoot osmotic potential at turgor loss, shoot pressure potential at full turgor, shoot relative water content at turgor loss, pre-dawn shoot water potential and midday shoot water potential were measured. In addition to the water relation parameters, growth and the amount of soluble carbohydrates were studied to gain a more complete understanding of how *C. stolonifera* seedlings are affected by NaCl during bud break. It was expected that the salt treatment would reduce growth, alter water relation parameters and disrupt carbohydrate metabolism. The effect of NaCl stress on cell wall elasticity and cell wall composition of shoot tissue was also examined; it was predicted that salt stress would alter both cell wall elasticity and cell wall composition, and that these changes would increase the salt tolerance of *C. stolonifera*.

4.2 Material and Methods

4.2.1 Plant Material

Cornus stolonifera seeds were collected from north of Fort McMurray, Alberta, Canada (57°1.67 N, 111°30.6 W). Seeds were stratified and planted following the same procedure as outlined in chapter 3 (section 3.2.1).

4.2.2 *Dormancy Induction*

Three-month-old *C. stolonifera* seedlings were transferred to a growth chamber to induce dormancy by reductions in temperature and photoperiod. The growth chamber conditions used to induce dormancy in *C. stolonifera* seedlings are shown in Table 4.1. The process took 12 weeks; at the end the seedlings had lost their leaves and were in a dormant state with no visible growth. After 12 weeks the seedlings were transferred to a cold room where they were maintained in the dark at 4°C for 5 weeks. During storage in the cold room the seedlings were watered approximately every 10 days to maintain moist, but not wet soil conditions. The seedlings had experienced the first step in cold acclimation, and were dormant; however maximum cold hardening was not achieved as the seedlings were not exposed to temperatures below freezing.

4.2.3 *Stress Treatments*

Dormant *C. stolonifera* seedlings were transferred to a hydroponic system as previously described (section 3.2.2). Two treatments, 0 and 50 mM NaCl, were replicated 5 times. Two experiments were done over time to ensure enough plant material was available for analysis of the cell wall composition. The same procedure was followed for each experiment. Twelve seedlings were placed in each container during the first experiment and 9 seedlings were used during the second experiment. Fewer seedlings were used for the second experiment because seedlings were not removed throughout the experimental period. A similar amount of root mass was present during both experiments. Most physiological parameters except for photosynthetic rates and water potential were measured on seedlings from the first experiment. Cell wall extraction, analysis of cell wall composition, photosynthetic rates and water potential measurements were done using seedlings from the second experiment.

Table 4.1 Growth chamber conditions used to induce dormancy in *C. stolonifera* seedlings.

Week	Photoperiod (hours of light)	Day temperature (°C)	Night temperature (°C)
1	15	20	15
2	12	20	15
3	10	20	10
4	10	20	10
5	8	15	10
6	8	15	10
7	8	15	5
8	8	10	5
9	8	10	5
10	8	8	5
11	8	5	5
12	Seedlings transferred to cold room, dark 24 hours at 4°C		

4.2.4 Growth

The height of 45 seedlings per treatment was measured after 7, 14, 21 and 30 days of treatment. Height was determined as the distance from the root collar to the tip of the newest leaves. After 32 days of treatment, seven plants per treatment per replicate were randomly selected to determine shoot and root dry weight. After the plants were harvested, the roots were rinsed and separated from the shoots and frozen in liquid nitrogen. Plant samples were stored at -80°C until lyophilized with a freeze dry system (Model 77520, Labconco Co. Kansas City, MO, USA). The root/shoot dry weight ratio was calculated.

The amount of new root growth was measured after 32 days of treatment on 15 seedlings per treatment. When the seedlings were harvested the new root growth was measured and an average value was recorded for each plant. The same 15 seedlings per treatment were used to determine the number of leaves per plant. The number of leaves that had a length greater than 5 cm was recorded to determine the size of the foliage.

4.2.5 Water Potential Measurements

Pre-dawn and midday shoot water potentials were determined 32 days after the beginning of the treatment with a pressure chamber operated at 0.02 MPa s^{-1} (model 1000, PMS Instruments, Corvallis, Oregon, USA). The top 6 to 10 cm of the shoot was cut and sealed in the pressure chamber with the cut end protruding. The shoot water potential was recorded when xylem sap was first observed on the cut end (Scholander et al. 1965). Pre-dawn measurements were done at least 30 minutes prior to sunrise on three randomly selected seedlings per treatment per replicate. Midday water potential measurements were done between 11:00 and 2:00 pm, on three randomly selected seedlings per treatment per replicate, or less if there were not sufficient seedlings available.

4.2.6 Photosynthesis, Pigment Analysis and Transpiration

The amount of chlorophyll a, chlorophyll b, total chlorophyll (a and b), the ratio of chlorophyll a/ chlorophyll b and amount of carotenoids in leaves of *C. stolonifera* seedlings were determined after 32 days of treatment. The procedure used to determine chlorophyll amounts is outlined in chapter three (section 3.2.5). In this experiment, the leaf disks were harvested and frozen at -80°C until lyophilized. The mass of the dry leaf disks was recorded before 80% acetone was added and final pigment amounts were expressed as mg g^{-1} dry weight. Photosynthesis was determined on 15 randomly selected seedlings per treatment 32 days after treatment. Refer to chapter three for the method used to determine photosynthetic rates (section 3.2.5).

4.2.7 Soluble Carbohydrates

Soluble carbohydrate extraction was done after 32 days of treatment. Fifteen seedlings per treatment were randomly harvested. The roots were rinsed and separated from the shoots at the root collar. Both roots and shoots were frozen in liquid nitrogen. The procedure followed to extract and quantify the amount of soluble carbohydrates has been previously outlined (section 3.2.6).

4.2.8 P-V Measurements

Cell wall elasticity and plant water relations were studied using the well-established pressure-volume (P-V) curve method (Scholander et al. 1965; Tyree and Hammel 1972; Cheung et al. 1975; Hinckley et al. 1980; Nabil and Coudret 1995; Renault and Zwiazek 1997). Pressure volume curves were constructed for 20 seedlings per treatment 32 days after treatment using the repeat pressurization method (Parker and Colombo 1995; Clifford et al. 1998) previously explained in chapter three (section 3.2.7).

4.2.9 Cell Wall Separation

Thirty-two days after the beginning of treatment, four plants per treatment were harvested for cell wall analysis. The roots were rinsed with deionized water, and

then the shoots and roots were separated at the root collar and frozen in liquid nitrogen. The samples were stored at -80°C before being lyophilized. Freeze dried tissues were ground using a Wiley mill (Arthur H. Thomas Co., Philadelphia, USA) with a 40 mesh screen. Two plants from each treatment per replicate were combined to ensure enough tissue was available for cell wall extraction. The cell walls were extracted from the shoot tissue following the protocol described by Zwiazek (1991), and previously described in section 3.2.8.1. The cell wall was then separated into a pectin, hemicellulose and cellulose fraction using the same procedure as outlined in section 3.2.8.2.

4.2.10 Analysis of Cell Wall Components

The pectin and hemicellulose fractions were analyzed by gas chromatography (GC) analysis, using the procedure described in section 3.2.9.1. The cellulose fraction was analyzed colorimetrically following the Updergraff procedure (Updergraff 1969) to determine the amount of glucose present in this fraction. The procedure used for cellulose analysis was the same as described in chapter three (section 3.2.9.2). The amount of lignin in the cell wall was determined colorimetrically from freeze dried cell wall material from the method outlined in Zwiazek (1991), originally developed by Morrison (1972). The procedure followed for lignin analysis is described in section 3.2.9.3.

4.2.11 Data Analysis

Data was analyzed with a general linear model (GLM) procedure using one way analysis of variance (ANOVA), using SPSS (Version 10.0, SPSS Inc., Chicago, USA). Statistically significant differences were determined at the 0.05 level.

4.3 Results

4.3.1 Growth

Although all seedlings broke dormancy, 16% of the seedlings treated with 50 mM NaCl had very little leaf expansion, as the initial leaves that appeared after bud break dried and no further leaf growth was observed. After 32 days of treatment, approximately 25% of seedlings treated with NaCl showed minor leaf injury, primarily as leaf curling and necrosis along the leaf margins, while no injury was observed in the untreated seedlings.

Shoot height of both the control and treated seedlings increased slowly during the first three weeks of treatment. After 30 days of treatment, seedlings treated with 50 mM NaCl were significantly shorter (13.7%) than the control seedlings (Figure 4.1). In addition, seedling shoot dry weight was significantly lower (38.8%) in seedlings treated with NaCl compared with the control seedlings (Table 4.2). Seedlings treated with NaCl for 32 days had 44.7% less foliage than the control seedlings (Table 4.3, Figure 4.2). Seedlings treated with NaCl had 50% fewer large leaves (length > 5 cm) compared to control seedlings; however the percentage of leaves greater than 5 cm in length was not significantly different between the control and treated seedlings (Table 4.3). The length of the new roots was 54.3% less in seedlings treated with NaCl compared with untreated seedlings (Table 4.3), while root dry weight was unaffected by the salt treatment (Table 4.2) The root/shoot dry weight ratio was significantly higher (29.7%) in seedlings treated with NaCl compared to untreated seedlings (Table 4.2).

4.3.2 Photosynthesis, Pigments and Transpiration

Stomatal conductance and transpiration rates of *C. stolonifera* leaves were significantly reduced in seedlings treated with 50 mM NaCl after 21 days (61.8% and 61.5% respectively) and 31 days (67.0% and 66.7% respectively) of treatment (Table 4.4). The amount of chlorophyll b was 21.3% lower, and the amount of carotenoids were 16.7% lower in the NaCl treated seedlings compared to the untreated

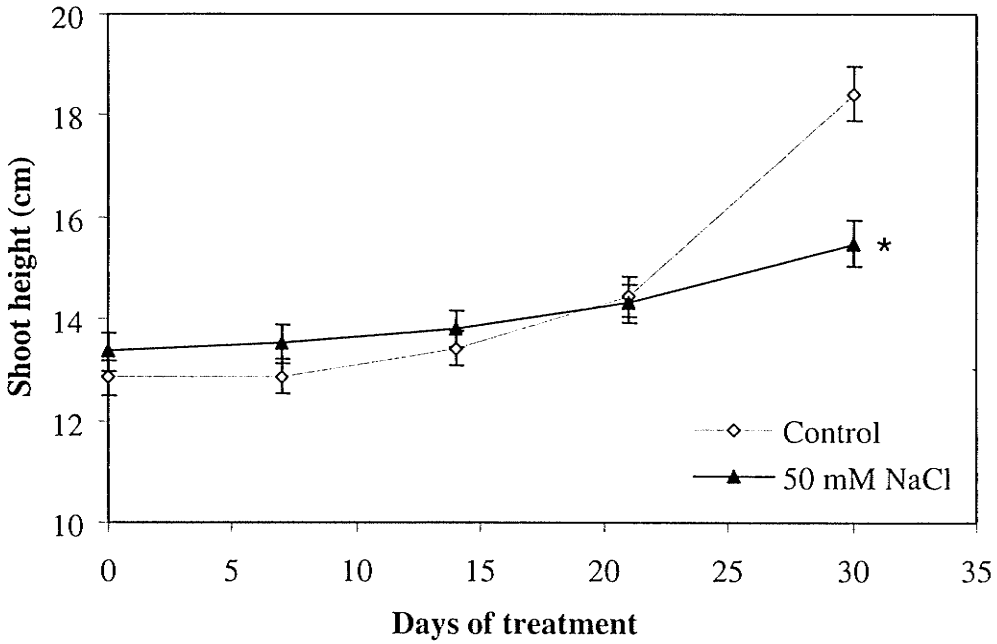

Figure 4.1 *Cornus stolonifera* shoot height measured during treatment with either 0 or 50 mM NaCl (mean ± SE, n = 45). * indicates a significant difference from the control after 32 days of treatment (p = 0.05).

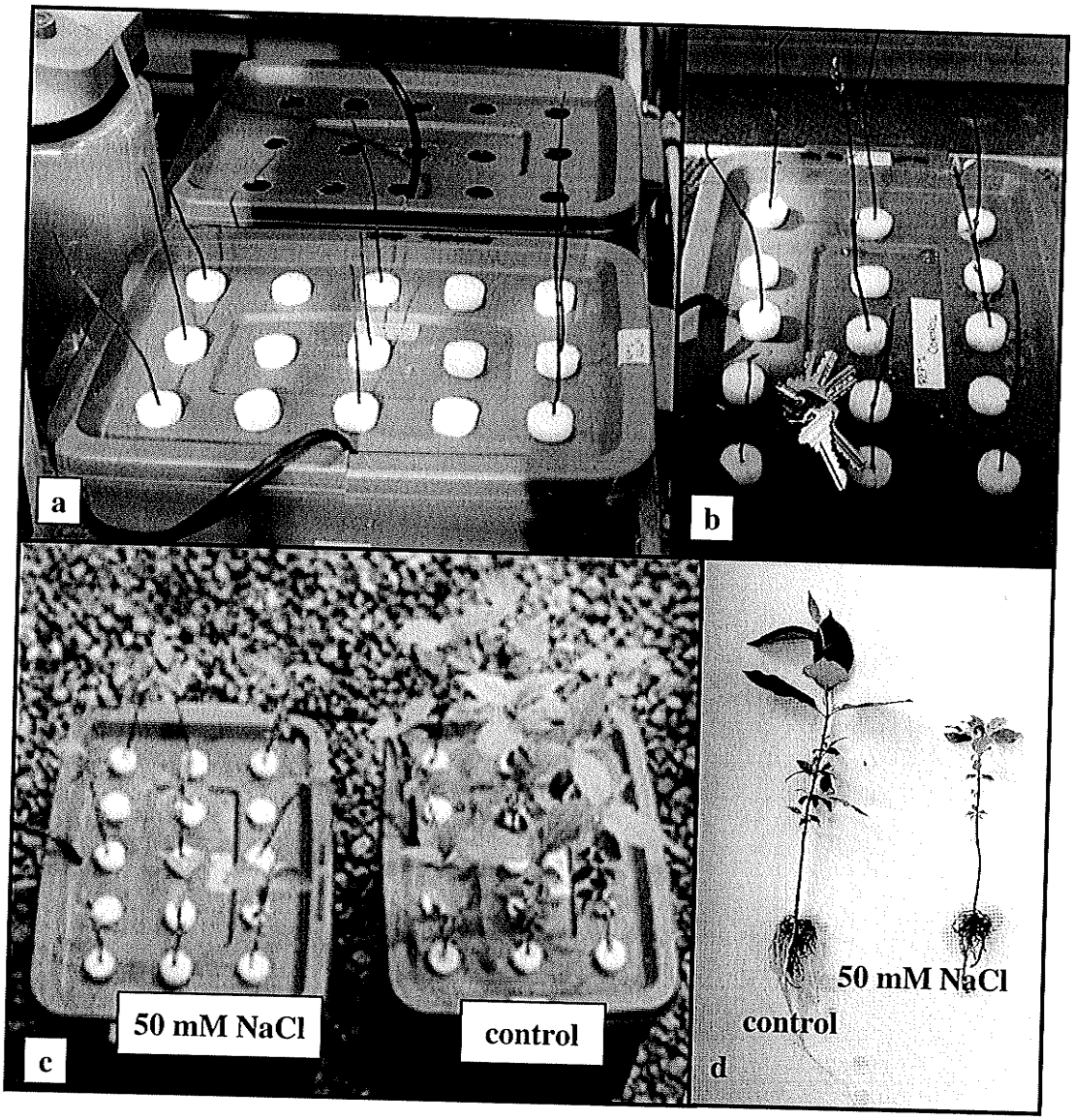

Figure 4.2 *Cornus stolonifera* seedlings, seedlings on first day of treatment (a); seedlings initiating growth (b); control and 50 mM NaCl treated seedlings after 32 days of treatment (c and d).

Table 4.2 *Cornus stolonifera* shoot and root dry weight and root to shoot ratio measured after 32 days of treatment with 0 or 50 mM NaCl (mean ± SE, n = 35) * indicates a significant difference from the control (p = 0.05).

Treatment	Root dry weight (g)	Shoot dry weight (g)	Root:shoot ratio
Control	0.30 (± 0.02)	0.67 (± 0.04)	0.45 (± 0.02)
50 mM NaCl	0.26 (± 0.01)	0.41* (± 0.02)	0.64* (± 0.02)

Table 4.3 Leaf number, leaves greater in length than 5 cm and length of new roots of *C. stolonifera* seedlings after 32 days of treatment with 0 or 50 mM NaCl (mean ± SE, n = 15). * indicates a significant difference from the control (p = 0.05).

Treatment	Number of leaves	Number of leaves > 5 cm	% of leaves > 5 cm	Length of new roots (mm)
Control	36.9 (± 3.6)	4.8 (± 0.5)	14.9 (± 1.7)	42.0 (± 2.5)
50 mM NaCl	20.4* (± 3.6)	2.4* (± 0.2)	16.6 (± 3.3)	19.2* (± 1.8)

Table 4.4 Transpiration rates and stomatal conductance of *C. stolonifera* seedlings after 21 and 31 days of treatment with 0 or 50 mM NaCl (mean ± SE, n = 15). * indicates a significant difference from the control (p = 0.05).

Days of treatment	Treatment	Stomatal conductance mmoles m^{-2} s^{-1}	Transpiration mmoles m^{-2} s^{-1}
21	Control	88.21 (± 5.47)	0.91 (± 0.93)
21	50 mM NaCl	33.70* (± 4.29)	0.35* (± 0.04)
31	Control	148.98 (± 17.82)	1.59 (± 0.17)
31	50 mM NaCl	49.10* (± 4.66)	0.53* (± 0.05)

seedlings (Table 4.5). The amount of chlorophyll a, total chlorophyll (chlorophyll a and chlorophyll b) and the ratio of chlorophyll a/ chlorophyll b were not significantly different from the control. The photosynthetic rate of seedlings treated with NaCl was significantly lower (37.0%) than the control seedlings (Table 4.5).

4.3.3 Water Relation Parameters and Cell Wall Elasticity

The amount of soluble carbohydrates was greater in the shoots than the roots of both treated and untreated seedlings (24.0% and 15.1% respectively), but no significant differences were found between the two treatments (Table 4.6). The bulk modulus of cell wall elasticity was 4.7 MPa in the shoot tissue of the control seedlings and 40.5% greater in seedlings treated with NaCl (Table 4.7), thus indicating salt stressed seedlings had more rigid cell walls than unstressed seedlings. The relative shoot water content at turgor loss was 94.21% and no difference was found between the treated and untreated seedlings (Table 4.7). The osmotic potential at full turgor was -1.08 MPa in the control seedlings and 9.25% less in seedlings treated with NaCl, but this difference was not significant (Table 4.7). The salt stressed seedlings had a 39.3% significantly greater pressure potential at full turgor compared to the control seedlings which had a pressure potential at full turgor of 0.68 MPa (Table 4.7). The osmotic potential at turgor loss in the control seedlings was -1.23 MPa, while the seedlings treated with NaCl had a significantly lower osmotic potential at turgor loss (21.2%) (Table 4.7). Pre-dawn and midday water potentials were -1.08 MPa and -0.56 MPa for the control seedlings respectively and no significant difference was found between the untreated and treated seedlings (Table 4.8).

Table 4.5 Pigment content (chlorophyll a, chlorophyll b and carotenoids) and photosynthetic rate determined at 400 μmoles photons m^{-2} s^{-1} of *C. stolonifera* seedlings after 32 days of treatment with either 0 or 50 mM NaCl (mean ± SE, n = 15). * indicates a significant difference from the control (p = 0.05).

Treatment	Photosynthetic rate (μmoles CO_2 m^{-2} s^{-1})	Chl a (mg g^{-1} DW)	Chl b (mg g^{-1} DW)	Chl a and b (mg g^{-1} DW)	Chl a/ Chl b ratio	Carotenoids (mg g^{-1} DW)
Control	3.05 (± 0.21)	2.65 (± 0.08)	0.80 (± 0.03)	3.45 (± 0.103)	4.31 (± 4.31)	0.12 (± < 0.01)
50 mM NaCl	1.92* (± 0.17)	2.11 (± 0.07)	0.63* (± 0.02)	2.74 (± 0.09)	4.34 (± 0.02)	0.10* (± < 0.01)

Table 4.6 Soluble carbohydrates of *C. stolonifera* seedlings measured after 32 days of treatment with either 0 or 50 mM NaCl (mean ± SE, n = 15). No significant differences were found between treatments.

Treatment	Roots (mg g^{-1} DW)	Shoots (mg g^{-1} DW)
Control	35.71 (± 1.67)	47.01 (± 2.76)
50 mM NaCl	36.53 (± 2.42)	43.01 (± 3.01)

Table 4.7 Bulk modulus of cell wall elasticity (ε) and water relation parameters (RWC_0, relative water content at turgor loss; ψ_{P100}, pressure potential at full turgor; $\psi_{\pi 100}$, osmotic potential at full turgor; $\psi_{\pi 0}$, osmotic potential at turgor loss) of *C. stolonifera* seedlings (mean ± SE, n = 15). Measurements were done after 32 days of treatment with 0 or 50 mM NaCl. * indicates a significant difference from the control (p = 0.05).

Treatment	ε (MPa)	RWC_0 (%)	ψ_{P100} (MPa)	$\psi_{\pi 100}$ (MPa)	$\psi_{\pi 0}$ (MPa)
Control	4.70	94.21	0.68	-1.08	-1.23
	(± 0.54)	(± 0.58)	(± 0.19)	(± 0.15)	(± 0.20)
50 mM NaCl	7.90 *	93.42	1.12 *	-1.19	-1.56 *
	(± 0.43)	(± 0.48)	(± 0.21)	(± 0.67)	(± 0.22)

Table 4.8 Pre-dawn and midday water potential (ψ) of *C. stolonifera* seedlings measured after 32 days of treatment with either 0 or 50 mM NaCl (mean ± SE, n = 14). No significant differences were found between treatments.

Treatment	ψ (MPa) pre-dawn	ψ (MPa) midday
Control	-1.08 (± 0.06)	-0.56 (± 0.02)
50 mM NaCl	-1.24 (± 0.08)	-0.62 (± 0.04)

4.3.4 Cell Wall Composition

The dry shoot tissue of untreated *C. stolonifera* seedlings contained 54.4% cell wall material. Seedlings treated with NaCl did not have a significantly different amount of cell wall material than the untreated seedlings (Table 4.9). The cell wall was composed of equal amounts of pectin and hemicellulose, each accounting for 27.4% of the cell wall in the untreated plants. There was no difference in either the amount of pectin or hemicellulose between the control and treated seedlings (Figure 4.3). The cellulose fraction of the untreated seedlings and treated seedlings contained 53.1% and 56.3% glucose respectively and was not significantly different. Control seedlings had 17.1% cellulose in their cell walls, and treated seedlings had slightly more (17.6%), but the difference was not significant (Figure 4.3). Lignin was not affected by the salt treatment and accounted for 18.0% of the cell wall in the control seedlings (Figure 4.3). The percent of cell wall that was lost during separation of the fractions was 5 to 10%.

The pectin fraction in shoot tissue of untreated *C. stolonifera* seedlings was composed primarily of arabinose (43.7%) with relatively large amounts of galacturonic acid (22.8%), galactose (16.5%) and glucose (12.7%), and minor amounts of rhamnose (4.3%). A significantly larger amount of glucose (17%) was found in the pectin fraction of seedlings treated with NaCl compared to untreated seedlings (Table 4.10). There was no significant difference between the treated and untreated seedlings with the amount of rhamnose, arabinose, xylose, galacturonic acid or galactose present in the pectin fraction. The seedlings treated with NaCl also contained a very small amount of xylose (0.5%). Xylose and glucose were the major polysaccharides present in the hemicellulose fraction of cell walls from shoot tissue of *C. stolonifera* seedlings. The hemicellulose fraction of untreated seedlings contained xylose (54.0%), glucose (24.0%), galactose (9.1%), mannose (2.9%), rhamnose (1.1%), fucose (1.0%), arabinose (0.8%) and galacturonic acid (0.8%). No significant differences were found between the treated and untreated seedlings in the composition of the hemicellulose fraction (Table 4.11).

Table 4.9 Percentage of cell wall extracted from shoot tissue of *C. stolonifera* seedlings after 32 days of treatment with 0 or 50 mM NaCl (mean ± SE, n = 10). No significant differences were found between treatments.

	Cell wall (% of shoot DW)
Control	54.4 (± 0.4)
50 mM NaCl	53.4 (± 0.5)

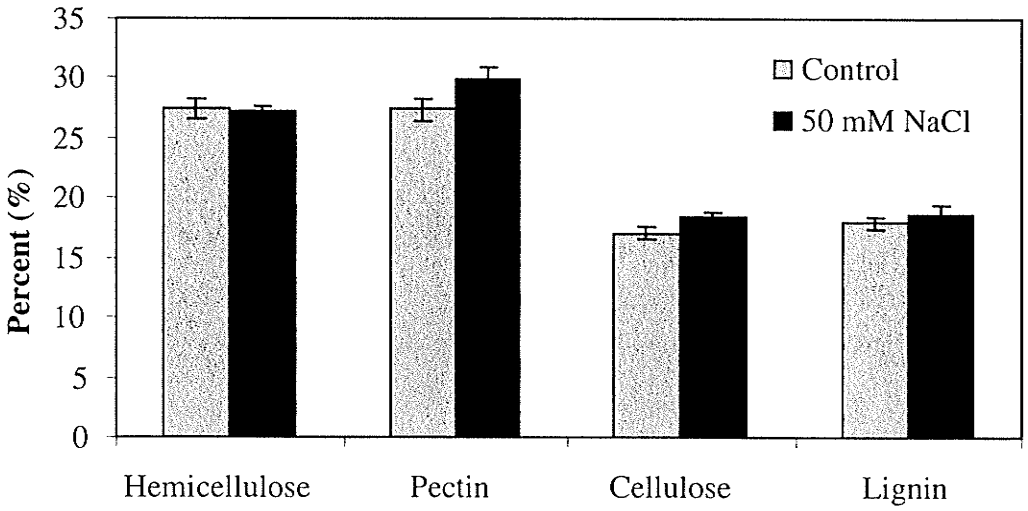

Figure 4.3 Cell wall composition of *C. stolonifera* shoot tissue after 32 days of treatment with either 0 or 50 mM NaCl (mean ± SE, n = 10). Results are expressed as a percentage of the total cell wall material extracted. The amount of cellulose presented was calculated from the amount of glucose present in the cellulose fraction. No significant differences were found between treatments. The percent of cell wall lost during separation of the fractions was 5-10%.

Table 4.10 Composition of the pectin fraction extracted from shoot cell walls of *C. stolonifera* seedlings, after 32 days of treatment with either 0 or 50 mM NaCl. Values are expressed as a percentage of all sugars identified in the pectin fraction (mean ± SE, n = 10). * indicates a significant difference from the control.

	Rhamnose (%)	Arabinose (%)	Xylose (%)	Galacturonic acid (%)	Galactose (%)	Glucose (%)
0 mM NaCl	4.3 (± 0.6)	43.7 (± 1.3)	0	22.8 (± 1.6)	16.5 (± 0.6)	12.7 (± 1.5)
50 mM NaCl	2.3 (± 0.7)	47.3 (± 0.9)	0.52 (± 0.4)	20.4 (± 1.0)	14.2 (± 0.5)	15.3* (± 1.3)

Table 4.11 Composition of the hemicellulose fraction extracted from shoot cell walls of *C. stolonifera* seedlings, after 32 days of treatment with either 0 or 50 mM NaCl. Values are expressed as a percentage of all sugars identified in the hemicellulose fraction (mean ± SE, n = 10). No significant differences were found between treatments.

	Rhamnose (%)	**Fucose (%)**	**Arabinose (%)**	**Xylose (%)**	**Galacturonic acid (%)**	**Mannose (%)**	**Galactose (%)**	**Glucose (%)**
Control	1.1	1.0	7.3	54.0	0.8	2.9	9.1	24.0
	(± 0.3)	(± 0.3)	(± 0.1)	(± 3.0)	(± 0.3)	(± 0.9)	(± 0.8)	(± 2.3)
50 mM NaCl	0.8	0.4	8.6	51.6	0.3	4.6	8.0	25.6
	(± 0.3)	(± 0.2)	(± 0.4)	(± 1.9)	(± 0.2)	(± 0.9)	(± 0.3)	(± 1.7)

4.4 Discussion

4.4.1 Growth

Cornus stolonifera seedlings treated with 50 mM NaCl during bud break had reduced shoot growth. Although leaf surface area was not directly examined in this study, the number of leaves and size of leaves were recorded and findings indicate that salt stressed *C. stolonifera* seedlings had a lower leaf surface area compared to unstressed seedlings. A reduction in leaf area was found for Acacia (*Acacia nilotica*) seedlings (Nabil and Coudret 1995) and bean (*Phaseolus vulgaris*) (Bray and Reid 2002) treated with NaCl. Despite the reduction in shoot growth and a reduction in new root growth no difference was found in the root dry weight between the treated and untreated seedlings. The results of this study are similar to what was previously found with actively growing *C. stolonifera* seedlings treated with NaCl (Chapter 3, section 3.2.1; Renault et al. 2000; Renault et al. 2001b). Typically root growth is less affected than shoot growth in plants grown under salt stress (Munns and Termat 1986; Shannon et al. 1994). In Acacia (*Acacia nilotica*), shoot dry weight was reduced by NaCl concentrations of 75 mM; however, root dry weight was not reduced until NaCl concentrations of 200 mM were used (Nabil and Coudret 1995). In sweet orange (*Citrus sinensis*) (Bañuls and Primo-Millo 1992) and poplar (*Populus deltoides*) (Fung et al. 1998) salt stress reduced shoot growth, but root growth was unaffected. An increase in the root/shoot ratio reduces the leaf area, but allows for the continued exploration and uptake of nutrients and water (Shannon et al. 1994). A reduction in shoot growth, particularly a reduction in leaf area, is considered beneficial to the plant as the transpiring surface is reduced, decreasing water requirements (Neumann 1995).

Despite the amount of work that has been done on a variety of species the reason for reduced growth under salt stress is not fully understood (Fung et al. 1998). Originally it was believed that during salt stress a reduction in turgor pressure prevented cell wall expansion (Singh et al. 1989; Neumann 1993; Neumann 1995). More recently it has been found that salt stressed plants often have a turgor pressure equal to or greater than unstressed plants (Iraki et al. 1989; Neumann 1993; Neumann

1995). As mentioned earlier the effect of salt stress on growth is very rapid and is quickly reversed when the stress is reduced; thus it is thought to be due primarily to osmotic stress causing changes in cell water relations (Munns and Termat 1986; Neumann 1993). The reduced new root growth in the salt treated seedlings may have reduced water uptake, further contributing to decreased shoot growth. An excess uptake of ions causing ion toxicity and ion imbalance (Greenway and Munns 1980) may also contribute to decreased growth as discussed previously in section 3.4.1.

Decreased cell wall expansion may also contribute to growth reduction during salt stress, but it varies with the species (Munns and Termat 1986). Reduced cell wall expansion due to cell wall hardening during salt stress has been found in a number, but not all species examined. Sodium chloride stress decreased cell wall extensibility in tobacco (*Nicotiana tabacum*) cells adapted to NaCl stress (Iraki et al. 1989) and chick-pea (*Cicer arietinum*) treated with NaCl (Muñoz et al. 1993). In maize seedlings (*Zea mays*), NaCl stress caused reduced growth due to a reduction in cell wall expansion in the leaf elongation zone (Neumann 1993). However, Cramer et al. (2001) did not show that cell wall hardening inhibited leaf elongation in salt stressed maize (*Zea mays*). In wheat (*Triticum aestivum*), water stress did not reduce the cell wall extensibility of treated seedlings compared to untreated seedlings (Wakabayashi et al. 1997).

A reduction in growth, particularly shoot growth may be due to root-to-shoot regulation with a growth regulator or hormone, such as cytokinin or abscisic acid (Munns and Termat 1986). Hormonal signals may have a greater influence on decreased growth than changes in cell water relations as salt stressed barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) plants did not show improved growth after the osmotic stress was artificially relieved by pressurizing the roots in a pressure chamber (Munns and Termat 1986). During bud break an increase in cytokinins and IAA occurred in *C. stolonifera* shoot tips and prior to root growth an increase in cytokinin and IAA, and a decrease in ABA occurred in the roots (Cappiello and Kling 1994). Changes in hormones initiate bud break and re-growth, but can also alter

growth rates during salt stress. Further work is recommended to examine the hormone activity during bud break in *C. stolonifera* seedlings under salt stress.

4.4.2 Photosynthesis, Pigments and Transpiration

Salt stress reduced photosynthetic rates in *C. stolonifera* during bud break. A reduction in photosynthesis may have contributed to decreased growth as less photosynthetic products were available. A reduction in photosynthesis is probably an effect of the salt stress caused either by ion toxicity or changes in the plant water relations. Reduced photosynthesis under salt stress has been previously found in actively growing seedlings treated with NaCl (section 3.4.2; Renault et al. 2001b). The chloroplast is the organelle considered to be the most affected by salt stress (Shannon et al. 1994). The chloroplast was affected in salt stressed *C. stolonifera* seedlings treated during bud break as the amount of chlorophyll b and the amount of carotenoids in the chloroplasts were reduced. These results differ from previous work with actively growing *C. stolonifera* that did not show a decrease in pigment contents when treated with NaCl (section 3.4.2, Renault et al. 2001b). Chlorophyll b is not considered to be essential for photosynthesis, but does improve the effectiveness of the light capturing complex (Nobel 1999). Decreases in the amount of chlorophyll b may contribute to the reduction in photosynthesis, but other factors are probably involved. Carotenoids function as accessory pigments in photosynthesis and are involved with the protection against damage caused by excited oxygen molecules, or free radicals (Nobel 1999). The decrease in carotenoids observed in *C. stolonifera* seedlings under salt stress may result in an increase in the number of free radicals which would further damage the chloroplast and affect the physiology of the seedling. The reduction in photosynthesis may also be due to ionic toxicity which may inhibit the light reaction or decreased carboxylase activity (Hagemeyer 1997). Photosynthesis may also be reduced due to negative feedback inhibition since reduced growth may reduce the sink (Herold 1980; Munns and Termat 1986). Transpiration and stomatal conductance were reduced in *C. stolonifera* seedlings treated with NaCl and this may be the primary reason decreased in photosynthetic rates were observed.

Increased stomatal resistance in *C. stolonifera* during salt stress is well supported through previous work with *C. stolonifera* (section 3.4.2, Renault et al. 1998; Renault et al. 2001b) and with other species such as radiata pine (*Pinus radiata*) (Sands and Clarke 1977). Increased stomatal resistance reduces water loss from the plant and limits the uptake of Na^+ and Cl^-. However, increased stomatal resistance also reduces CO_2 availability for photosynthesis and is not a sustainable long-term salt tolerance strategy (Hasegawa et al. 2000). Despite the long-term problems associated with increased stomatal resistance it is a regular occurrence in salt stressed plants and may be an effect of the stress rather than a tolerance mechanism. More work is recommended to fully understand the implications of decreased transpiration in *C. stolonifera* seedlings during salt stress.

4.4.3 Water Relation Parameters and Cell Wall Elasticity

Midday and pre-dawn shoot water potentials of *C. stolonifera* seedlings were not altered by salt stress. These results vary from a number of other species for which a decrease in plant water potential occurred during salt stress. A decrease in leaf water potential was observed in both salt sensitive and salt tolerant olive (*Olea europaea*) cultivars treated with 100 mM NaCl (Gucci and Tattini 1997), and in Acacia (*Acacia nilotica*) seedlings treated with 75 mM NaCl (Nabil and Coudret 1995). Pre-dawn leaf water potentials were significantly reduced in poplar (*Populus deltoides*) stressed with 85 mM and 170 mM NaCl compared with untreated seedlings (Fung et al. 1998). During salt stress a decrease in the plant water potential benefits the plant as water uptake can be maintained (Greenway and Munns 1980). Despite evidence from other species supporting a reduction in leaf water potential during NaCl stress, previous work with *C. stolonifera* has yielded variable results. Salt stress has resulted in an increase, decrease as well as not altering the shoot water potential depending on the stress treatment (section 3.4.3, Renault et al. 1998; Renault et al. 1999; Renault et al. 2001a), suggesting that the shoot water potential of *C. stolonifera* is not affected below a certain threshold of salt stress.

A reduction in the plant water potential is often achieved during salt stress by a reduction in the solute potential due to osmotic adjustment. Although salt stress did

not affect the shoot osmotic potential at full turgor, the osmotic potential at turgor loss was lower in *C. stolonifera* seedlings treated with NaCl during bud break than in the control seedlings. At turgor loss the solutes present in the cytoplasm are concentrated as the water content decreases. The reduction in osmotic potential at turgor loss may indicate a low level of osmotic adjustment that could not be detected at full turgor. Two Acacia (*Acacia nilotica*) cultivars that are both considered to be moderately salt tolerant differed in their response to 100 mM NaCl. Both the osmotic potential at turgor loss and at full turgor were reduced in one cultivar (*cupressiformis*), but only the osmotic potential at full turgor was reduced in the other cultivar *(tomentosa)* (Nabil and Coudret 1995). In mangrove seedlings (*Avicennia germinans*) treated with seawater, the decrease in osmotic potential at full turgor and turgor loss was attributed to intracellular solute accumulation (Suarez and Sobrado 1998).

Supporting the absence of an effect of salt stress on the osmotic potential at full turgor in shoot tissue of *C. stolonifera* was the absence of soluble carbohydrate accumulation. Soluble carbohydrates accumulate under salt stress and contribute to osmotic adjustment in wheat (*Triticum aestivum*) (Kerepesi et al. 1998), jack pine (*Pinus banksiana*), and white spruce (*Picea glauca*) (Koppenaal et al. 1991). Changes in the amount of soluble carbohydrate present in plant tissue not only occur during salt stress but also vary with plant stage of development. In the fall an increase in the amount of soluble sugars occurs in the stems of *C. stolonifera* along with a breakdown of starch (Ashworth et al. 1993). During winter dormancy soluble sugars levels are highest, and in the spring the amount of soluble carbohydrates decrease in *C. stolonifera* seedlings (Ashworth et al. 1993). Sucrose content decreased in the shoot tip of *C. stolonifera* seedlings during bud break (Cappiello and Kling 1994). The changes occurring in soluble carbohydrate accumulation during bud break may be sufficiently large enough that changes in soluble carbohydrate accumulation due to salt stress could not be detected in *C. stolonifera*. Further investigation into the accumulation of soluble carbohydrates or other compatible osmolytes, such as proline, glycine-betaine, mannitol, and sorbitol (Volkmar and Steppuhn 1998), is

recommended as a significant decrease in the osmotic potential at turgor loss occurred in *C. stolonifera* seedlings treated with NaCl.

The shoot pressure potentials at full turgor were significantly higher and the cell walls were more rigid in *C. stolonifera* seedlings treated with NaCl, compared to unstressed seedlings. The combination of an increased pressure potential and a more rigid cell wall does not indicate that these differences would benefit the seedling during salt stress. During osmotic stress rigid cell walls are thought to contribute to tolerance as they would allow for a greater decrease in pressure potential for a given decrease in water content (Bowman and Roberts 1985). This would allow plants with rigid cell walls to quickly decrease their water potential and maintain water uptake. In mangrove seedlings (*Avicennia germinans*) (Suárez and Sobrado 2000) and alligator weed (*Alternanthera philoxeroides*) (Bolaños and Longstreth 1984), increased cell wall rigidity helped to maintain a gradient between the plant and rhizosphere water potential that allowed for continued water uptake during NaCl stress. Despite the evidence that supports increased cell wall rigidity contributes to stress tolerance, it is not always found. In loblolly pine (*Pinus taeda*) drought stress caused a decrease in cell wall elasticity; however, this decrease was not thought to increase drought tolerance as shoot pressure potential increased (Meier et al. 1992). Similarly, the decreased cell wall elasticity observed in *C. stolonifera* seedlings treated with NaCl was not found to benefit the plant during salt stress as the pressure potential increased and the plant water potential was not affected. Differences observed in cell wall elasticity between the treated and untreated seedlings may be due to the reduced growth in the salt stressed seedling resulting in differences in tissue and cell types that would alter the tissue elasticity, or may simply be an effect of the salt stress.

No change in the relative water content at turgor loss occurred for shoot tissue of *C. stolonifera* seedlings treated with NaCl during bud break, but stress reduced the relative water content at turgor loss in a number of other species. In white spruce (*Picea glauca*) (Marshall and Dumbroff 1999) and lemon (*Citrus limon*) (Ruiz-Sánchez et al. 1997) drought stress reduced the relative water content at turgor loss of needles and leaves respectively. In olive (*Olea europaea*) seedlings treated with NaCl,

the leaf relative water content decreased compared to untreated seedlings in both salt tolerant and salt sensitive cultivars (Gucci and Tattini 1997). A reduction in the relative water content at turgor loss indicates that other changes, such as increased cell wall elasticity, are occurring to maintain turgor at the lower relative water content. An increase in cell wall elasticity was found in drought stressed olive (*Olea europaea*) (Gucci and Tattini 1997) and white spruce (*Picea glauca*) (Marshall and Dumbroff 1999), indicating the cell wall could shrink around the protoplast as the water content decreased.

4.4.4 Cell Wall Composition

The total amount of cell wall material in shoot tissue of *C. stolonifera* treated with NaCl during bud break was not affected by the stress. This finding is similar to what was found for actively growing seedlings (section 3.4.4). Not only was the amount of total cell wall material not affected in *C. stolonifera* seedlings during bud break in this study but, the amount of hemicellulose, cellulose, pectin and lignin extracted from the cell wall were also not affected. This result differs from what was found for actively growing *C. stolonifera* seedlings as an increase in hemicellulose was found in the salt stressed seedlings (section 3.4.4). The effect of salt and osmotic stress on cell wall composition varies depending on the species. For example in wheat (*Triticum aestivum*) coleoptiles, the hemicellulose fraction increased and the cellulose fraction decreased (Wakabayashi et al. 1997). In spruce (*Picea glauca*), the pectin fraction increased, but the cellulose content was not affected (Zwiazek 1991). The amount of lignin decreased in rice (*Oryza sativa*) (Lin and Kao 2001) and Chenopodiaceae (*Atriplex proxtrate*) (Wang et al. 1997) during salt stress, while lignin was not affected by salt stress in tomato (*Lycopersicon esculentum*) roots (Peyrano et al. 1997). This variability in the effect of salt stress on cell wall composition may depend on a number of factors including but not limited to species, tissue, duration of the stress, and previous life history as *C. stolonifera* seedlings treated with NaCl when actively growing (section 3.4.4) were found to have an increase in the amount of hemicellulose present in the cell wall.

The composition of the hemicellulose fraction of *C. stolonifera* seedlings during bud break was unaffected by salt stress. This result is supported by Zwiazek (1991) who found no changes in the hemicellulose composition of white spruce (*Picea glauca*) seedlings after drought stress. These results are also similar to what was found for actively growing seedlings (section 3.4.4). The composition of the pectin fraction was affected by NaCl stress as the amount of glucose increased in *C. stolonifera* seedlings treated with NaCl. This differs from what was found for actively growing *C. stolonifera* seedlings where the pectin fraction was not affected by salt stress. The results of this study also differ from drought stressed white spruce (*Picea glauca*) seedlings where a decrease in the amount of glucose was found in the stressed seedlings (Zwiazek 1991). In the halophyte sea star (*Aster tripolium*), the pectin fraction of salt stressed plants contained a higher amount of arabinose and a lower amount of glucose compared with unstressed plants (Binet 1985) suggesting these changes may increase salt tolerance. Pectin functions in regulating cell wall porosity and pH modulation of the cell wall (Buchanan et al. 2000). Changes in pectin binding along with an increase in glucose may alter these properties, increasing or decreasing stress tolerance. These findings indicate that the effect of stress on the amount of glucose in the pectin fraction may vary with species, and/or the type of stress. The methods used to extract, and then separate the pectin fraction also differed between studies and it is possible that during this study not all starch was removed from the shoot tissue of salt stressed *C. stolonifera* seedlings. Starch accumulation may have occurred, artificially inflating the amount of glucose present in the pectin fraction of salt stressed seedlings. Further work should be done to study starch accumulation under salt stress, as well as further investigation into the structure and linkage of pectin molecules.

A period of dormancy had little effect on the cell wall as the composition was very similar in both *C. stolonifera* seedlings that experienced dormancy and younger seedlings that had never been dormant. These findings may indicate the cell wall structure in *C. stolonifera* is conserved and not easily altered by stress. However, future work should be done to look at the changes in cell wall composition that might

occur during cold acclimation to sub-zero temperatures and how this would affect subsequent stress tolerance.

4.5 Summary

Salt stress did not prevent *C. stolonifera* seedlings from breaking dormancy, but it did reduce both shoot height and dry weight. Other effects of the salt stress included reduced photosynthetic rates, reduced amounts of chlorophyll b and carotenoids, and increased stomatal resistance. Cell wall elasticity was lower in salt stress seedlings, but the water potential was unaffected and the pressure potential increased. These changes suggested that alterations in cell wall elasticity were not a salt tolerance mechanism. Cell wall composition was mildly affected by NaCl. The amount of glucose increased in the pectin fraction. The seedlings used for this study were dormant, but not fully cold acclimated. Although a period of dormancy did not improve the stress tolerance of *C. stolonifera* it is recommended work be done to look at the salt tolerance of seedlings that have been fully cold acclimated.

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5.0 Conclusions

Sodium chloride decreased transpiration and photosynthetic rates while it did not alter shoot water relations or cell wall elasticity in actively growing *C. stolonifera* seedlings. Shoot growth was reduced in actively growing seedlings after 35 days of treatment with 50 mM NaCl, as a reduction in shoot dry weight occurred. Neither height nor root dry weights were reduced by the stress, indicating only a minor effect of NaCl on growth. These results support previous work that has found *C. stolonifera* to be relatively salt tolerant (Renault et al. 2000). The absence of an effect of NaCl stress on cell wall elasticity suggests that elastic adjustment is not used as a salt tolerance mechanism in shoot tissue of actively growing *C. stolonifera* seedlings when treated with concentrations of up to 50 mM NaCl. Osmotic adjustment in the shoot tissue was also not found to contribute to salt stress tolerance, as the shoot osmotic potential was not altered by the salt stress. The hemicellulose fraction of the cell wall increased in salt stressed seedlings, and this increase could contribute to salt tolerance as an increase in the amount of hemicellulose may increase cell wall hardening helping to maintain turgor (Neumann 1995). In addition, salt stress did not affect the amount of cellulose, lignin or pectin in the cell wall, or the composition of these fractions. Although this study examined osmotic adjustment, it was preliminary work and more work should be done to fully understand osmotic adjustment in *C. stolonifera* as solutes other than carbohydrates may accumulate in plant tissue. The role that increased hemicellulose may play in salt stress tolerance has been previously mentioned (Zwiazek 1991), but it is not fully understood and more work will need to be done to fully understand the implications. This study focused mainly on shoot tissues. It is also recommend that more work be done examining the effects of NaCl stress on root physiology and morphology. Changes in the root osmotic potential, water potential, and cell wall elasticity may contribute to increased tolerance. Further investigation into the control of ion movement through the roots should also be done as previous work indicated *C. stolonifera* is able to control the movement of ions from the roots into the shoots (Renault et al. 2001).

Cornus stolonifera seedlings treated with 50 mM NaCl during bud break experienced moderate salt stress, as both shoot height and dry weight were significantly reduced. The lower cell wall elasticity in the salt stressed seedlings was not found to contribute to salt tolerance. The difference in cell wall elasticity was attributed to differences in tissue type as a result of decreased growth, but may also simply be an effect of the stress. The amount of pectin, hemicellulose, cellulose and lignin in the cell wall was not affected by NaCl, while the pectin fraction did contain a greater amount of glucose. Soluble carbohydrates were did not accumulate in either root or shoot tissue. However, the osmotic potential at turgor loss was found to be more negative in *C. stolonifera* seedlings treated with NaCl, indicating osmotic adjustment may occur. Therefore, more work is recommended to determine if compatible osmolytes play a role in the salt tolerance of *C. stolonifera.* More work should also be done to determine the influence that changes in the pectin composition, such as increased levels of glucose, may have on salt stress tolerance.

Although the effects of NaCl on actively growing and dormant seedlings could not be statistically compared the results suggest that NaCl stress affects *C. stolonifera* seedlings more during bud break than when the seedlings are actively growing. This work suggests actively growing seedlings are more suited for reclamation work. However, more work should be done to examine the effects of salt stress on *C. stolonifera* seedlings during bud break that have experienced true cold acclimation. The seedlings were treated hydroponically during this experiment over a relatively short time period. A longer-term study under field conditions is recommended to gain a better understanding of how *C. stolonifera* seedlings tolerate saline conditions. The effects of stress on plants and the mechanisms of stress tolerance are difficult to study as there are numerous physiological and metabolic processes involved, and findings can be interpreted in various ways. Changes seen in salt treated seedlings may be alterations that will contribute to increased tolerance and benefit the plant, but they may also be an effect of the salt stress.

5.1 References

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Appendix

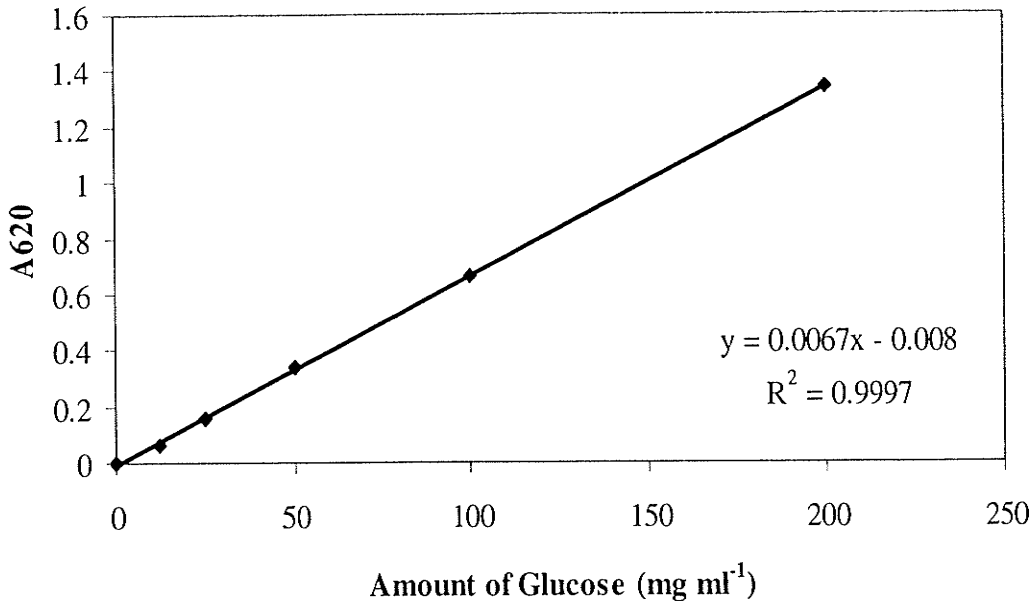

Figure A1 Standard curve for the Anthrone test. Absorbance was read at 620 nm, standard curve is based on D(+) glucose extracts.

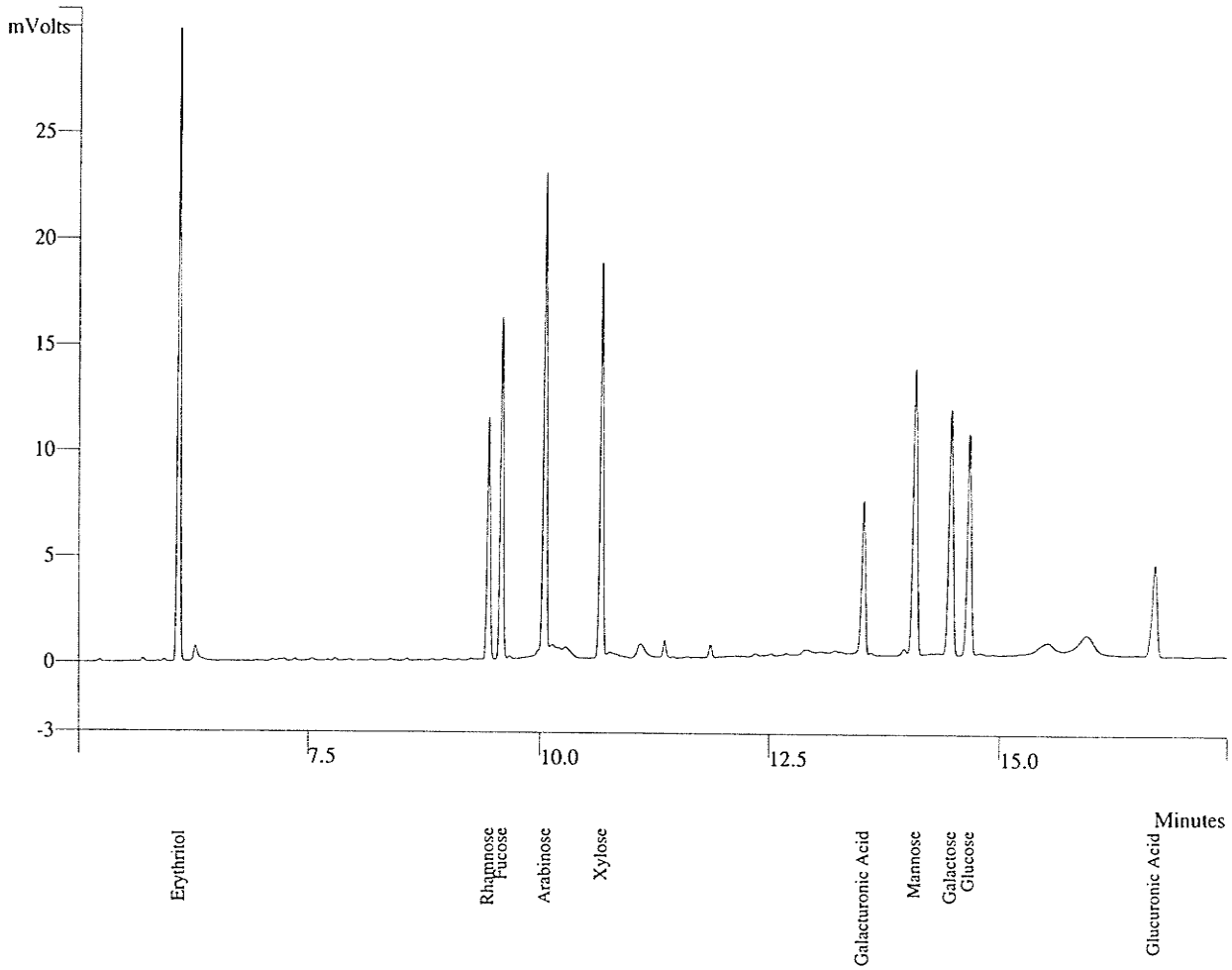

Figure A2 Chromataograph of known sugars, used to determine retention times.

Table A3 ANOVA for *C. stolonifera* seedling height (cm), after 0, 14 and 28 days (TIME) of treatment with either 0, 25 or 50 mM NaCl (TRT).

Days of treatment	Source	df	Mean square	F	Sig.
0	TRT	2	0.103	0.087	0.919
14	TRT	2	44.974	1.257	0.377
28	TRT	2	47.088	3.458	0.134
overall	TRT	2	71.406	3.655	0.125
	TIME	2	9650.778	68.964	0.001
	TRTxTIME	4	21.933	1.290	0.351

Table A4 ANOVA for shoot dry weight (g) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7, 21, 28 or 35 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.154	5.975	0.063
21	TRT	2	0.365	5.726	0.067
28	TRT	2	0.070	0.211	0.818
35	TRT	2	3.931	10.365	0.025
overall	TRT	2	1.206	10.693	0.025
	TIME	3	17.867	12.808	0.005
	TRTxTIME	6	1.337	5.803	0.005

Table A5 ANOVA for root dry weight (g) of seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7, 21, 28 or 35 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.0131	1.809	0.276
21	TRT	2	0.06929	2.105	0.237
28	TRT	2	0.0180	0.305	0.753
35	TRT	2	0.140	3.706	0.120
overall	TRT	2	0.019	0.312	0.748
	TIME	2	1.758	9.403	0.011
	TRTxTIME	6	0.07828	3.017	0.049

Table A6 ANOVA for root/shoot dry weight (g) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7, 21, 28 or 35 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.001	0.191	0.834
21	TRT	2	0.006	1.431	0.340
28	TRT	2	0.002	0.305	0.753
35	TRT	2	0.014	3.663	0.123
overall	TRT	2	0.018	1.667	0.297
	TIME	2	0.029	10.734	0.008
	TRTxTIME	6	0.002	0.494	0.801

Table A7 ANOVA of photosynthesis (μmoles CO_2 m^{-2} s^{-1}) and pigment content (mg g^{-1} DW) for *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 32 days of treatment.

Parameter	Source	df	Mean square	F	Sig.
Photosynthesis	TRT	2	1.475	8.356	0.037
Chlorophyll a	TRT	2	1.063	3.127	0.152
Chlorophyll b	TRT	2	2.432	1.325	0.362
Total Chlorophyll	TRT	2	1.52	2.843	0.171
Chl a/Chl b ratio	TRT	2	0.406	0.7	0.549
Carotenoids	TRT	2	0.002	2.116	0.236

Table A8 ANOVA of transpiration (mmoles m^{-2} s^{-1}) and stomatal conductance (mmoles m^{-2} s^{-1}) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 28 days of treatment.

Parameter	Source	df	Mean square	F	Sig.
Transpiration rate	TRT	2	8.886	22.389	0.007
Stomatal conductance	TRT	2	66406.656	34.358	0.003

Table A9 ANOVA of soluble carbohydrates (mg g^{-1} DW) for *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 21 days of treatment.

Tissue	Source	df	Mean square	F	Sig.
Shoot	TRT	2	582.721	1.424	0.341
Root	TRT	2	0.860	0.018	0.982

Table A10 ANOVA for bulk cell wall elastic modulus, ε (MPa) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7 and 21 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.974	0.426	0.680
21	TRT	2	0.128	0.082	0.923
overall	TRT	2	0.877	4.361	0.082
	TIME	1	0.495	0.275	0.652
	TRTxTIME	2	0.228	0.062	0.941

Table A11 ANOVA for osmotic potential at full turgor, $\Psi_{\pi 100}$ (MPa) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7 and 21 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.01688	2.480	0.194
28	TRT	2	0.002149	0.069	0.934
overall	TRT	2	0.008	0.336	0.733
	TIME	1	<0.001	0.152	0.734
	TRTxTIME	2	0.012	0.824	0.501

Table A12 ANOVA for osmotic potential at turgor loss, $\psi_{\pi 0}$ (MPa) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7 and 21 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.018	2.347	0.202
28	TRT	2	0.006	0.119	0.891
overall	TRT	2	0.010	0.209	0.820
	TIME	1	0.003	0.110	0.771
	TRTxTIME	2	0.016	0.938	0.463

Table A13 ANOVA for pressure potential at full turgor, ψ_{P100} (MPa) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7 and 21 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	0.006	0.250	0.790
28	TRT	2	0.035	0.443	0.669
overall	TRT	2	0.023	0.314	0.747
	TIME	1	0.002	0.076	0.809
	TRTxTIME	2	0.021	0.560	0.610

Table A14 ANOVA for relative water content at turgor loss, RWC_0 (%) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 7 and 21 days (TIME) of treatment.

Days of treatment	Source	df	Mean square	F	Sig.
7	TRT	2	9.601	2.441	0.203
28	TRT	2	1.510	0.614	0.586
overall	TRT	2	6.859	2.802	0.173
	TIME	1	30.978	3.980	0.184
	TRTxTIME	2	4.252	1.077	0.422

Table A15 ANOVA of amount of cell wall in dry shoot tissue (%) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 21 days of treatment.

Source	df	Mean square	F	Sig.
TRT	2	8.299	0.453	0.665

Table A16 ANOVA of cell wall fractions (% of total cell wall) extracted from shoot tissue of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 21 days of treatment.

Fraction	Source	df	Mean square	F	Sig.
Hemicellulose	TRT	2	25.037	3.358	0.139
Pectin	TRT	2	7.226	4.824	0.086
Lignin	TRT	2	0.997	0.174	0.846
Cellulose	TRT	2	2.347	0.594	0.594

Table A17 ANOVA of carbohydrate composition of the cell wall pectin (%) of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 21 days of treatment.

Carbohydrate	Source	df	Mean square	F	Sig.
Rhamnose	TRT	2	18.244	4.373	0.186
Arabinose	TRT	2	3.850	0.153	0.867
Galacturonic Acid	TRT	2	64.575	1.299	0.435
Mannose	TRT	2	79.687	2.726	0.268
Galactose	TRT	2	25.007	10.082	0.090
Glucose	TRT	2	69.882	0.524	0.656

Table A18 ANOVA of carbohydrate composition of hemicellulose (%) extracted from shoot tissue cell walls of *C. stolonifera* seedlings treated with either 0, 25 or 50 mM NaCl (TRT) after 21 days of treatment.

Carbohydrate	Source	df	Mean square	F	Sig.
Rhamnose	TRT	2	0.001	0.002	0.998
Fucose	TRT	2	0.122	1.000	0.500
Arabinose	TRT	2	0.940	0.550	0.645
Xylose	TRT	2	57.399	0.225	0.816
Mannose	TRT	2	0.105	1.000	0.5
Galactose	TRT	2	1.765	2.401	0.294
Glucose	TRT	2	24.906	0.100	0.909

Table A19 ANOVA of height (cm) for *C. stolonifera* seedlings during bud break after 0, 7, 14, 20 and 30 days (TIME) of treatment with either 0 or 50 mM NaCl (TRT).

Days of treatment	Source	df	Mean square	F	Sig.
0	TRT	1	5.457	0.626	0.473
7	TRT	1	9.474	1.452	0.295
14	TRT	1	3.442	0.345	0.588
20	TRT	1	0.441	0.036	0.858
30	TRT	1	193.600	9.004	0.040
overall	TRT	1	10.615	0.196	0.681
	TIME	4	228.754	123.1	<0.001
	TRTxTIME	4	50.415	44.404	<0.001

Table A20 ANOVA of dry weights (g) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Parameter	Source	df	Mean square	F	Sig.
Shoot DW	TRT	1	1.139	9.892	0.35
Root DW	TRT	1	0.027	1.328	0.313
Root/Shoot	TRT	1	0.585	39.235	0.003

Table A21 ANOVA of growth parameters for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Parameter	Source	df	Mean square	F	Sig.
Root growth (cm)	TRT	1	145.200	11.741	0.027
# of leaves	TRT	1	2033.633	12.186	0.025
# of leaves > 5 cm	TRT	1	43.200	38.687	0.003
% of leaves > 5 cm	TRT	1	22.862	0.84	0.411

Table A22 ANOVA of stomatal conductance (mmoles m^{-2} s^{-1}) for *C. stolonifera* seedlings during bud break after 21 and 31 days (TIME) of treatment with either 0 or 50 mM NaCl (TRT).

Days of treatment	Source	df	Mean square	F	Sig.
21	TRT	1	22299.226	48.935	0.002
31	TRT		74820.108	16.578	0.015
overall	TRT	1	89406.092	28.964	0.006
	TIME	1	21770.721	9.408	0.037
	TRTxTIME	1	7713.241	4.098	0.113

Table A23 ANOVA of transpiration (mmoles m^{-2} s^{-1}) for *C. stolonifera* seedlings during bud break after 21 and 31 days (TIME) of treatment with either 0 or 50 mM NaCl (TRT).

Days of treatment	Source	df	Mean square	F	Sig.
21	TRT	1	2.388	26.904	0.007
31	TRT	1	8.369	21.844	0.009
overall	TRT	1	9.849	42.969	0.003
	TIME	1	2.754	7.303	0.054
	TRTxTIME	1	0.908	3.742	0.125

Table A24 ANOVA of photosynthesis (μmoles CO_2 m^{-2} s^{-1}) and pigment content (mg g^{-1} DW) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Parameter	Source	df	Mean square	F	Sig.
Chlorophyll a	TRT	1	1.391	7.372	0.053
Chlorophyll b	TRT	1	0.141	8.892	0.041
Chlorophyll a and b	TRT	1	2.416	7.816	0.049
Chlorophyll a/ chlorophyll b ratio	TRT	1	0.006	0.222	0.662
Carotenoids	TRT	1	0.003	8.939	0.040
Photosynthesis	TRT	1	0.948	22.691	0.009

Table A25 ANOVA of water relation parameters for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Parameter	Source	df	Mean square	F	Sig.
ε (MPa)	TRT	1	84.640	9.059	0.039
ψ_{P100} (MPa)	TRT	1	1.920	11.741	0.026
$\psi_{\pi 100}$ (MPa)	TRT	1	0.168	2.409	0.188
$\psi_{\pi 0}$ (MPa)	TRT	1	1.016	13.251	0.021
RWC_0 (%)	TRT	1	0.001	0.953	0.383
Pre-dawn ψ (MPa)	TRT	1	0.097	4.054	0.177
Midday ψ (MPa)	TRT	1	0.027	0.896	0.397

Table A26 ANOVA of soluble carbohydrates (mg g^{-1} DW) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Tissue	Source	df	Mean square	F	Sig.
Shoots	TRT	1	119.630	1.063	0.361
Roots	TRT	1	4.988	0.124	0.742

Table A27 ANOVA of amount of cell wall in dry shoot tissue (%) of *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Source	df	Mean square	F	Sig.
TRT	1	4.835	3.985	0.117

Table A28 ANOVA of cell wall fractions (% of cell wall) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Cell wall fraction	Source	df	Mean square	F	Sig.
Pectin	TRT	1	< 0.001	0.328	0.597
Hemicellulose	TRT	1	<0.001	0.055	0.825
Cellulose	TRT	1	9.850	7.804	0.049
Lignin	TRT	1	1.782	0.317	0.603

Table A29 ANOVA of carbohydrate composition of cell wall pectin (%) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Carbohydrate	Source	df	Mean square	F	sig.
Rhamnose	TRT	1	20.122	4.306	0.106
Arabinose	TRT	1	39.102	2.224	0.207
Xylose	TRT	1	2.071	0.945	0.386
Galacturonic acid	TRT	1	22.191	1.517	0.283
Galactose	TRT	1	80.871	6.843	0.056
Glucose	TRT	1	24.783	3.285	0.139

Table A30 ANOVA of carbohydrate composition of cell wall hemicellulose (%) for *C. stolonifera* seedlings during bud break after 32 days of treatment with either 0 or 50 mM NaCl (TRT).

Carbohydrate	Source	df	Mean square	F	Sig.
Rhamnose	TRT	1	0.333	0.831	0.414
Fucose	TRT	1	1.488	1.807	0.250
Arabinose	TRT	1	8.160	4.548	0.100
Xylose	TRT	1	29.161	1.982	0.232
Galacturonic Acid	TRT	1	0.900	0.518	0.511
Mannose	TRT	1	15.868	3.626	0.130
Galactose	TRT	1	5.431	2.397	0.197
Glucose	TRT	1	13.220	3.284	0.144