Physiological and Biochemical Characterization of Root Cell Wall in *Suaeda salsa* and *Spinach oleracea* under Saline Condition

(塩分条件下における *Suaeda salsa* と *Spinach oleracea* の根細胞壁の生理と生化学的特性)

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Contents

Chapter 1 General Introduction

Approximately 800 million hectares of land throughout the world are affected by salinity and sodicity. Much of this salinity is natural, but irrigation practices have also contributed to the extensive salinization and degradation of land in many regions. Of the 230 million hectares of irrigated land, 45 million hectares are affected by salt, representing approximately 20% of the total (FAO, 2005). Addressing the spread of salinization and increasing the salt tolerance of crops are critical global challenges (Munns 2002).

1.1 Salt tolerance mechanisms in plants

Salts are mainly derived from irrigation water and seawater ((Flowers and Yeo 1995). Increased soil salinity exposes plants to excessive ionic sodium (Na^+) and chloride (Cl^-) to a cascade of responses in the plant (Julkowska and Testerink 2015, Munns and Gilliham 2015). Previous studies have shown that photosynthesis, gas exchange, water (and ion) relations, and osmotic adjustment are inhibited by salt stress(Rengasamy, Chittleborough and Helyar 2003, Koyro 2006, Koyro and Eisa 2008, Munns 2002). Plant evolution has resulted in a large array of mechanisms to tolerate the stresses associated with increased soil salinity. The salinity decreases the ability of a plant to take up water, and once Na^+ and Cl^- are taken up in large amounts by roots, both Na^+ and Cl^- inhabit the plant growth by impairing metabolic processes and decreasing photosynthetic efficiency (Flowers and Yeo 1995, Mäser, Gierth and Schroeder 2002). Under nonsaline conditions, the cytosol of higher plant cells contains about 100 mM K^+ and less than 10 mM Na^+, an ionic environment in which enzymes are optimally functional. In saline environments, cytosolic Na+ and $Cl^–$ increase to more than 100 mM, and these ions become cytotoxic. High concentrations of salt cause protein denaturation and membrane destabilization by reducing the hydration of these macro-molecules. However, Na^+ is a more potent denaturant than K^+. At high concentrations, apoplastic Na^+ also competes for sites on transport proteins that are necessary for high-affinity uptake of K^+. The effects of high salinity in plants occur through a two-phase process: a fast response to the high osmotic pressure at the root–soil interface and a slower response caused by the accumulation

of Na^+ (and Cl^-) in the leaves (Munns and Tester 2008). In the osmotic phase there is a reduction in shoot growth, with reduced leaf expansion and inhibition of lateral bud formation. The second phase starts with the accumulation of toxic amounts of Na^+ in the leaves, leading to the inhibition of photosynthesis and biosynthetic processes. Although in most species Na^+ reaches toxic concentrations before Cl^-, some plant species, such as citrus, grapevine, and soybean, are highly sensitive to excess Cl^-. Under salinity stress, salt-tolerant plants enact mechanisms to alleviate osmotic stress by reducing water loss while increasing water uptake. In addition, the salt-tolerant plants can minimize the harmful effects of ionic Na^+ stress by the exclusion of Na^+ from leaf tissues and by compartmentalization of Na^+, mainly into vacuoles ((Munns and Tester 2008, Blumwald 2000). The sodium exclusion and sequestration consume more energy to activate the proton pump or active the SOS1 exchanger protein, resulting in the overproduction and accumulation of reactive oxygen species (ROS) under saline conditions, which causes significant damages to the key macromolecules and cellular structures in the meristem (Shi et al. 2000, Zhu et al. 2016).

1.2 Growth responses of different species to salinity

Salt-tolerant plants genetically adapted to salinity are termed halophytes (from the Greek word halo, “salty”), while less salt-tolerant plants that are not adapted to salinity are termed glycophytes (from the Greek word glyco, “sweet”). Halophytes and glycophytes differ greatly in their growth response to salinity. Halophytes have high salt tolerance, which can complete the life cycle in a salt concentration of at least 200 mM NaCl under conditions similar to those that might be encountered in saline soils (Flowers and Colmer 2008). For the glycophytes, the growth of roots is disrupted when soil salinity exceeds 4 dS/m (equivalent to about 40 mM NaCl). The study of the mechanisms of salt tolerance and physiological response to salinity between the glycophytes and halophytes is a useful basis for screening and breeding for higher salt tolerance and yield (Koyro and Eisa 2008). As so far, studies on the mechanism of salt tolerance of halophytes have been mainly focused on the cell membrane and vacuole (Meychik, Nikolaeva and Yermakov 2006). However, none of these approaches have resulted in truly tolerant crops in the fields, and even the best performing genotypes led to a 50% of yield loss when grown under saline conditions

(Munns et al. 2012).

1.3 Cell wall composition responses of different species to salinity

So far, considerable attention has been paid to the protoplast of the plants. As the outmost layer of the root, the cell wall may also play an important role against the salinity stress, which highly regulates the cell expansion in the meristem. However, the study on the functions of root cell wall under salinity stress has been ignored (Tenhaken 2015).

All plant cells are surrounded by an extensible primary cell wall, which contains cellulose, pectin and hemicellulosic polysaccharides. Pectin affects the pH, ion homeostasis, and water permeability of the cell wall. The cellulose fibrils are cross-linked by hemicelluloses and pectin, serve as a scaffold for the binding of other wall components. Hemicellulose polysaccharides are complex molecules that associate with cellulose microfibrils, providing a cross-linked matrix. The chemical composition of root cell wall also affects the physical characteristics of root: The pectin polysaccharides determines wall porosity; Cellulose determines the hardness of cell wall, which is related to the growth of a cell; The cross-linking glycan related to the elasticity and extensibility of cell wall, which affects cell elongation and expansion.

Changes occur in the chemical composition of root cell walls in response to salt treatments. For instance, Jbir et al.（2001) reported that cell-wall peroxidase activity increased and with its lignification in wheat. The structural arrangement of cellulose microfibrils in sorghum (*Sorghum bicolor L.*) epidermal cell walls changed in response to salt treatments, becoming less parallel, and invagination in these walls increases (Koyro 1997). In cotton (*Gossypium hirsutum L.*), the amount of cellulose decreased and the amount of uronic acid increased in response to salt treatments (ZHONG and LAUCHLI 1993). Root tip pectin content in soya bean (*Glyxine max*) decreased (An et al. 2014) and Casparian bands in the maize *(Zea mays L.*) endodermis developed closer to the root tip in response to salt treatments (Chen et al. 2016).

The characteristics of the root cell wall in ion-binding and ion transport depend

on the chemical properties of the cell wall, which is closely related to the chemical compositions. The cell wall polysaccharides participate in wall resistance in terms of sequestration of Na^+ and maintain NaCl tolerance in *Madia sativa* (Shoresh, Spivak and Bernstein 2011). Gonzalez et al. (Gonzalez, Syvertsen and Etxeberria 2012) reported that cell wall within the stele acted as Na^+ traps and the immobilization of Na^+ by cell walls was key contributing mechanisms enabling citrus leaves to maintain lower levels of Na^+. Plant cell wall acts as supplier of K and Na ions, which induced resistance to fungal stress in pea and cowpea (Amano et al. 2013). However, it is still unknown how much Na^+ could be trapped in the apoplast or whether this would make a significant contribution to sequestering Na^+ in root tissue or affect accumulation in the shoot. At present, there are few reports detailing how compositional variation in different cell wall polymers influences Na^+ binding, or how this would influence K^+ passage or the binding and passage of Ca^{2+} in roots.

The cell wall composition is different among plant varieties. The proportion of different cell wall components can change in response to exposure to salt. There are notable differences in cell wall composition between different cell types and different species which might reflect different strategies to cope with excess salt. Therefore, measurements of changes in root cell wall components relating to growth rate in response to salt stress need to be assayed to study the relation between chemical composition of root cell wall and root elongation under saline condition.

1.4 Responses of cell wall extensibility to salinity for different species

In cell growth, cell wall extensibility is critical for controlling cell extension and morphogenesis(Wu and Cosgrove 2000, Cosgrove 2005). Salt stress causes significant wall stiffness resulting in detrimental effect on root growth (An et al. 2014, Munns and Tester 2008). The increase in cell wall extensibility may benefit to root elongation under saline condition.

The root growth is more sensitive to salinity in the early stage, which is pivotal for plant establishment (Ashraf and Waheed 1990, Shalhevet, Huck and Schroeder 1995). In *S. salsa*, a halophyte, the early germination stage was the most sensitive and reduction in growth with increase in salinity (Duan et al. 2007, Song et al. 2011). In

the early stage, salinity stress causes significant cell wall stiffness and the reduction of turgor pressure resulting in the detrimental effect on the root growth (An et al. 2014, Munns and Tester 2008). In the primary cell walls of the roots, cell wall extensibility is critical for controlling cell growth and morphogenesis, providing resistance against turgor pressure and constraining the uniaxial growth of roots (Cosgrove 2005).

Root growth depends on cell division and cell elongation. Cell-wall extensibility in root elongation zone is one of the most important growth-limiting factors. Increased cell wall extensibility is of benefit to the root elongation under abiotic stress. Extensibility of the cell wall is regarded as an important factor in the regulation of cell elongation in plant tissues (Kojima et al. 1991). The maize seedlings adapted to low water potential by making the walls in the apical part of the root more extensible (Wu and Cosgrove 2000). The Na accumulation in the root cell interferes pectin cross-linking and reduces the stabilizing influence of pectin in the cell wall. The decrease of pectin fraction in elongation zone leads to the reduction of cell wall extensibility, further inhibited root elongation(An et al. 2014).

There are two components of wall extensibility: a viscosity or plastic component and an elastic component. The viscosity component is the ability of the wall to be deformed irreversibly in a time-dependent manner; the elastic component is instant deformation and reversible after the deforming force is removed. The cell elongation is accompanied by an increase in cell wall extensibility that is regulated by the two physical parameters, i.e. viscosity coefficient and elasticity modulus. To understand the effect of NaCl concentration on the viscoelastic extensibility of the cell wall, the physical parameters of three elastic (E_0, E_1, E_2) and three viscous (η_N, η_1, η_2) parameters were measured and analyzed by using a Kelvin-Voigt-Burgers' model. This method was developed for plant roots by Tanimoto et al. (2000). Although the physiological functions of the viscoelastic parameters (E_0, E_1, E_2, η_N η_1, η_2) in creep-extension analysis are not fully understood, some characteristics of these parameters has been elucidated. For example, low-pH treatment in vitro predominantly decreased the viscosity coefficients rather than the elastic moduli (Tanimoto et al. 2000). Changes in these parameters were also reported to correlate with the elongation growth of sorghum roots that had been treated with or without silicon in vivo (Hattori et al. 2003). Thus the changes of these parameters are possibly regulating factors of elongation zone of roots both in vitro and in vivo.

1.5 Responses of pectin characteristics to salinity for different spinach cultivars

The plant cell wall is essential for the strength, growth, and development of the plant (Caffall and Mohnen 2009). It is primarily made up of pectin, hemicellulose, and cellulose. Pectin consists of galacturonic acid as its main building block, and is present in the homogalacturonan (HG) and rhamnogalacturonan I (RG-I) structural elements. While the HG backbone is composed only of galacturonic acid residues, the RG-I backbone has alternating rhamnose and galacturonic acid residues. The rhamnose residues in RG-I, a structural element of pectin, can be replaced with neutral sugar side chains made up of arabinose and galactose (Voragen et al. 2009). Hemicelluloses are composed of xylans, xyloglucans, and mannans (Scheller and Ulvskov 2010). Xyloglucan, the major hemicellulosic polysaccharide in primary plant cell walls, has a cellulose-like backbone that is branched at the O-6 position by xylosyl residues. These xylose units can be substituted by other monosaccharides such as galactose, fucose, and arabinose. Cellulose consists of a linear chain composed of β-(1 → 4)-linked glucose residues (Scheller and Ulvskov 2010).

The main changes in the cell wall following salinity stress were found in the pectin sugar composition, pectin characteristics such as HG:RG-I and the degree of methyl esterification (Corrêa-Ferreira et al. 2019, Huang et al. 2017). Salt stress have been shown to increase the amount of carboxylated polysaccharides in the cell walls of rice (*Oryza sativa*) and coffee plants (*Coffea arabica*)(Aquino, Grativol and Mourão 2011, de Lima et al. 2014). Treatment of red-osier seedling shoot cell walls with 50 mM NaCl resulted in a decrease in the galacturonic acid content and an increase in rhreamnose content of the extracted pectin (Mustard and Renault 2004). HG:RG-I ratio increased in Artemisia annua, indicating increase of HG domain of pectins from plants submitted to salt stress (Caffall and Mohnen 2009). The HG domain of pectin has been shown to control the viscosity and mechanical properties of the cell wall matrix, and changes in its structure may affect plant development. It has been suggested that negatively charged cell wall polysaccharides may increase the Donnan potential, facilitating ion transport from the cell at high salt concentrations, or slow the movement of Na^+ towards the cells. However, the relation between pectin

characteristics and physical properties of root cell wall and root elongation under saline condition are largely unknown.

1.6 *Suaeda salsa* and *Spinacia oleracea*

A halophyte (*Suaeda salsa*) and a glocophyte (*Spinacia oleracea*), both of which belong to same family, were chosen as experimental materials. By comparing the responses of each species to salinity and the different responses between these two species, we may further understand the salinity tolerance mechanisms which resides in plants.

Suaeda salsa: This plant belongs to Amaranthaceae family, most of the plants in this family show high tolerance to various environmental conditions and distributes widely in Europe and Asia with potential economic values for food and oil production. It grows both in inland saline soils and intertidal zones. This species is a promising model for understanding salt tolerance mechanism (Song et al. 2009). Thus, this plant was taken as the "tolerance species" in this study. The salt tolerance mechanism of this species has been found to be ion accumulation (citation). The toxic ions are sequenced into vacuoles while retain large amount of water also in vacuoles to dilute ion concentration.

Spinacia oleracea: This plant belongs to the same family as *S. salsa*, but its salinity tolerance is much lower than *S. salsa*. In this study, *S. oleracea* was taken as "sensitive species" for comparative analysis. The salinity tolerance mechanisms of *S. oleracea* is reported to depend on the selective absorption of K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-} and depressed absorption of Na^+ and Cl^+. Because of ion binding sites in cell walls, ion transport may be affected by root cell wall in *S. oleracea.*

1.7 Objectives

The objectives of my phD study were therefore:

(1) To investigate the effects of sodium chloride (NaCl) on cell wall compositions and cell wall extensibility.

(2) To study the relation between chemical composition and physical properties of root cell wall and root elongation under a saline condition.

(3) To elucidate the relation between pectin characteristics and physical

properties of root cell wall and root elongation under a saline condition.

Chapter 2 Cell Wall Components and Extensibility Regulate Root Growth in *Suaeda salsa* and *Spinacia oleracea* under Salinity

2.1. Introduction

Soil salinity is an environmental factor that severely limits global agricultural productivity. Understanding the mechanisms underlying salt tolerance would contribute to global crop production (Munns and Gilliham 2015). The growth response of halophytes and glycophytes to salinity can differ significantly. Halophytes show high salt tolerance and can complete their life cycle under salt concentrations that reach 600 mM NaCl (Rozema and Schat 2013). In contrast, the root growth of glycophyte is severely hindered when the concentration exceeds 70 mM NaCl (ZHAO et al. 1999). The study of the physiological responses of glycophytes and halophytes to salinity is useful for understanding the mechanisms underlying plant responses to salinity. Recent studies on the mechanism of salt tolerance in plants have focused on the symplast pathway (Isayenkov and Maathuis 2019),while studies on the apoplast pathway are relatively limited.

The cell wall matrix is composed of three types of polysaccharides: pectin, hemicellulose, and cellulose. The structure, function, composition, and linkage of cell wall components have been reviewed by Cosgrove (2018). However, less is known about the salt-tolerance-related functions of root cell walls, which directly interact with salts present in the soil solution and in the plant. Saline treatment changes the chemical composition of glycophyte root cell walls. For example, sodium ions can displace Ca^{2+} from their binding sites on the cell wall, which can reduce pectin-crosslinking and cell wall integrity (Feng et al. 2018, Hocq, Pelloux and Lefebvre 2017, O'Neill et al. 2004, Proseus and Boyer 2012). Furthermore, the cellulose content can decrease when uronic acid increases in cotton grown in saline conditions (ZHONG and LAUCHLI 1993). Conversely, although pectin content decreases in apical root tips of soybean uder saline conditions, cellulose content increases (An et al. 2014). In *Artemisia annua*, the monosaccharide composition of pectin and hemicellulose was altered in response to salt stress (Corrêa-Ferreira et al. 2019).

Similarly, salinity increased the concentration of carboxylated polysaccharides in *Oryza sativa* (Aquino et al. 2011). The cell wall of stele cells in citrus plants can also influence root Na^+ transport by acting as an Na^+ trap (Ricardi et al. 2014). Furthermore, the Na^+ concentration in the cortical cell walls of a salt-sensitive barley cultivar was approximately twice that observed in the cortical cell walls of a salt-tolerant cultivar, indicating that cell wall composition can influence Na^+ transport (Flowers and Hajibagheri 2001).

Previous studies have demonstrated that the mechanical and chemical characteristics of the root cell wall are associated with root growth under abiotic stress conditions (Munns 2002, Neumann, Azaizeh and Leon 1994). The cell wall's extensibility is an important trait in the regulation of cell elongation under different abiotic stress conditions (Cosgrove 2005, Wu and Cosgrove 2000). Plastic deformability in the root elongation zone of soybean roots is reduced by salt stress (Nonami et al. 1994), and the extensibility of root cell walls is inhibited by water deficit in maize and damask rose (Al-Yasi et al. 2020, Fan et al. 2006). Furthermore, the changes in cell wall extensibility are associated with root elongation in sorghum plants treated with silicon (Hattori et al. 2003); SiO_2 treatment increases cell wall pectin content and physical strength (Cui et al. 2020). In contrast, cell wall extensibility and root elongation are inhibited in maize by the binding of Al^{3+} to the cell wall (Ma et al. 2004) and cause the accumulation of cell wall polysaccharides (Tabuchi and Matsumoto 2001). Similarly, lead (Pb) accumulates mainly in the roots, where it has a high affinity for galacturonic acid (Połeć-Pawlak et al. 2007), which may change pectin structure and decrease cell wall extensibility. Finally, Pb has been found to reduce cell wall extensibility by influencing the synthesis of cell wall polysaccharides (Hossain et al. 2015).

Despite this wealth of information, there are no reports of changes in the interactions between the mechanical properties of root cell walls and root growth or between the chemical composition of root cell walls and root growth under saline conditions. Therefore, we investigated cell wall composition, extensibility, and viscosity in the root elongation zone of young seedlings of a halophyte (*S. salsa* L.) and a glycophyte (*S. oleracea* L.), which both belong to the Amaranthaceae family (while they were formerly grouped to Chenopodiaceae). These plants were grown

under different NaCl concentration so we could compare the response of each species to salinity to understand the mechanisms of plant salt tolerance better.

2.2. Material and Methods

2.2.1. Plant Materials

Seeds of *S. salsa* were collected in the coastal saline region of Bohai Bay, Hebei Province, China. The seeds of *S. oleracea* 'Akinokagayaku' were bought in a local seed market. Although they belong to the same family, *S. oleracea* is sensitive to salinity stress, indicated by its relatively low ability to counteract salinity by salt exclusion (Muchate et al. 2019). In contrast, *S. salsa* is relatively more tolerant to salt stress than *S. oleracea* due to its ability to accumulate large concentrations of salts in its vacuoles (Song et al. 2009).

2.2.2. Seedling Growth and Salt Treatment

Seeds of *S. salsa* were surface sterilized with 5% NaClO for 10 min and then thoroughly rinsed with water. Seed germination and seedling growth were conducted in growth chambers (MLR-350HT; Sanyo, Osaka, Japan). Temperature and relative humidity of the chambers were 20 °C and 70%, respectively. Approximately 15–20 seeds were aligned on a side of a filter paper and placed in a zip-lock plastic bag. The filter paper was rinsed every day with 1/12 diluted Hoagland solution (pH 6.5) during the two-day germination period for *S. salsa*, and over a four-day period for *S. oleracea*. The seeds were germinated in the dark. After germination, the roots of the seedlings were rinsed every 2 d with 1/12 diluted Hoagland solution mixed with 0, 100, 200, and 300 mM NaCl. The seedlings were subjected to the salinity treatments for 8 d. Light conditions were set to a 12 h/12 h light/dark cycle. Each salt concentration treatment group included 24 filter paper germination sheets. After germination completed, five filter paper sheets were randomly selected at 2 d intervals from each treatment to measure the root length of each seedling. As we found in the preliminary experiment, 1/12 diluted Hoagland solution did not cause nutrient deficiency, and exposure to high salt concentration did not cause growth arrest

through osmotic shock. Therefore, we chose 1/12 diluted Hoagland solution to reveal the salt tolerance of these species and their differences when grown in graduated low nutrient conditions.

2.2.3. Mechanical Parameters of the Root Cell Wall

Root samples from separately cultured seedlings were excised from the apical zone (extending 15 mm behind the root tip) and immediately transferred to boiling methanol in a water bath heated to approximately 80 °C. Samples were incubated in this solution for 5 min. Methanol-killed root segments were rehydrated with 1/12 diluted Hoagland solution (pH 6.5), and cell wall elastic moduli and viscosity coefficients were measured using a creep meter (RE2-33005C-1,2; Yamaden, Tokyo, Japan). This 2–5 mm root segment from the apical zone was fixed between the two clamps of the creep meter, and a tensile force along the direction of root elongation of 0.025 to 0.1 N was applied to root samples depending on the measured diameter. We dropped a drip of water at the basis of root section before the extension measurement and kept a humid environment around the creepmeter using a humidifier. The extension process was electronically recorded at intervals of 0.5 s for 300 s. The load was then released, and root shrinkage was recorded for 300 s. The difference in length between the maximum extension after 300 s and the final length after 600 s was reported as a reversible extension (i.e., viscoelastic extension), while the difference between final and original lengths (3 mm) was reported as a plastic extension. The elastic modulus (E_0) and viscosity coefficient (η_N) (Tanimoto et al. 2000) were determined using the creep meter software supplied. We measured 15 root segments from each NaCl treatment.

2.2.4. Isolation of Root Cell Wall Polysaccharides

Seedlings were prepared as described above. Seedling roots were thoroughly washed and cut into two 5 mm segments. The 0–5 mm segment measured from behind the root cap was considered the elongation zone (Nonami and Boyer 1990), and the 5–10 mm segment measured from behind the root cap were sampled. Approximately 96–120 segments from six filter paper sheets were collected as one replicate, and each treatment had four replicates.

Cell wall polysaccharides were isolated, as previously described (Tanimoto and Huber 1997). Briefly, the root segments were homogenized for 30 s in a mixture of ice-cold Tris-HCl buffer (pH 7.4) and Tris buffer-saturated phenol using a bead crusher (Model μT-12; TAITEC Co., Ltd., Tokyo, Japan). The homogenate was centrifuged (Kubota 6200; KUBOTA Corporation Co., Ltd., Tokyo, Japan) for 10 min at 4 °C, and the supernatant was discarded. The pellet containing the cell walls was further purified by sequential incubation and centrifugation in ethanol (thrice), acetone (twice), a mixture of methanol and chloroform (1:1, *v*/*v*) (thrice), and again in acetone and ethanol. Cell walls were treated with 0.2 mg mL^{-1} pronase in 0.05 mM phosphate buffer (pH 7.0) containing 5% ethanol for 16 h at 30 °C. After centrifugation, the supernatant was discarded.

Cell wall polysaccharides were extracted as described by An et al. (An et al. 2014). Briefly, the pectin fractions were extracted five times with CDTA at pH 6.5 at 20 °C. To extract the remaining polyuronides, cell walls were further extracted three times with CDTA at 100 °C (hot CDTA) for 1 h each. These CDTA extractions were designated as the pectin fraction. Hemicellulose I and II were sequentially extracted with 1 M and 4 M KOH, respectively. These extractions in KOH solutions were repeated three times for 4, 16, and 4 h each, respectively. Residual alkaline-insoluble precipitates were designated as the cellulose fraction, which was dissolved in a small amount of 72% (*v*/*v*) sulfuric acid for 1 h and diluted with distilled water to measure total sugars. The amounts of uronic acid and total sugars in each extract were measured using the *m*-hydroxydiphenyl colorimetric (Ahmed and Labavitch 1978) and phenol-sulfuric acid (Dubois et al. 1951) methods, respectively.

2.2.5. Statistical Analysis

All data were analyzed using the analysis of variance (ANOVA) and correlation; means were compared using Tukey's Honestly Significant Difference test ($p < 0.05$). All statistical analyses were performed using SPSS software version 28.0 (SPSS, Inc., Chicago, IL, USA).

2.3. Results

2.3.1. Root Growth

Figure 2-1 shows the root length and salt resistance coefficient of the two species when treated with different salt concentrations for 8 d after germination. Root growth of *S. oleracea* seedlings was inhibited by salinity and almost ceased in plants treated with 200 mM NaCl. Conversely, root growth was not inhibited in *S. salsa* plants treated with either 100 mM or 200 mM NaCl. Salinity had a significant negative effect on root growth with 300 mM NaCl in the halophytic species *S. salsa*. The inhibition was more pronounced in *S. oleracea* than in *S. salsa*.

2.3.2. Mechanical Properties of Root Cell Wall

Figure 2-2 shows E_0 and η_N recorded for the root cell walls in the 2–5 mm root tip zone in *S. salsa* and *S. oleracea* seedlings growing under different NaCl concentrations. Lower values of E_0 indicate higher cell wall elasticity, while greater values of η_N indicate higher cell wall viscosity (Tanimoto et al. 2000). E_0 and η_N in *S. oleracea* root tips increased with increasing salinity, whereas 100 mM NaCl treatment reduced E_0 but 300 mM NaCl treatment raised it for *S. salsa*. In all treatments, E_0 was higher in *S. salsa* than in *S. oleracea*. η_N was higher in *S. salsa* than in *S. oleracea* under 0 and 300 mM NaCl treatments, whereas the opposite was observed in the 100 and 200 mM NaCl treatments.

The regression of root length on E_0 revealed significant negative correlations for each species (Figure 2-3). The effect of E_0 on root growth was considerably more pronounced in *S. oleracea* than in *S. salsa*. Similarly, the regression of root length on η_N also showed negative correlations for the two species, and again, the effect of η_N was more pronounced in *S. oleracea* than in *S. salsa* (Figure 2-3). In *S. oleracea*, the viscoelastic extension was reduced by salt treatments. In *S. salsa*, the plastic extension showed negative values, except in the 200 mM treatment. The negative value of plastic extension means that the root shrunk even shorter than the original length after extension. In contrast, this phenomenon was not found in *S. oleracea* (Figure 2-4).

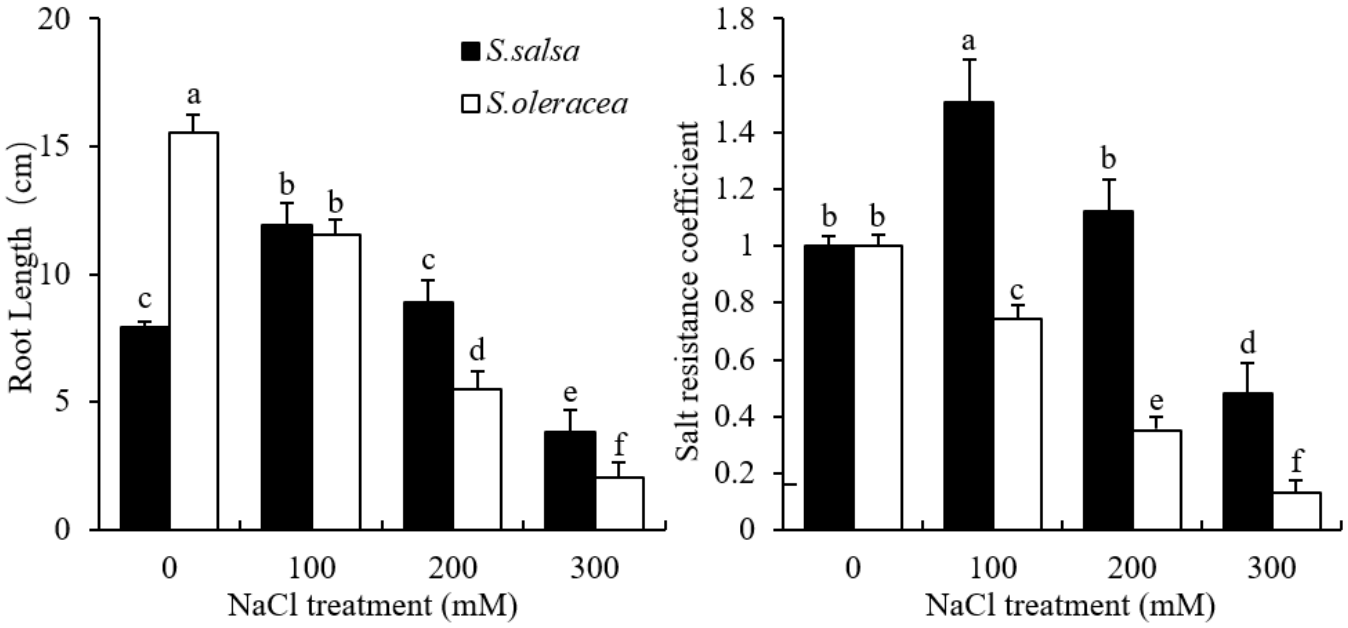

Figure 2-1. Final root growth and salt resistance coefficient of *S. salsa* and *S. oleracea* in 0, 100, 200, and 300 mM NaCl treatments. Data are mean + S.D. (n = 5). Salt resistance coefficient was calculated as: root length of NaCl treatments/root length of 0 mM NaCl. Different letters indicate significant differences ($p < 0.05$).

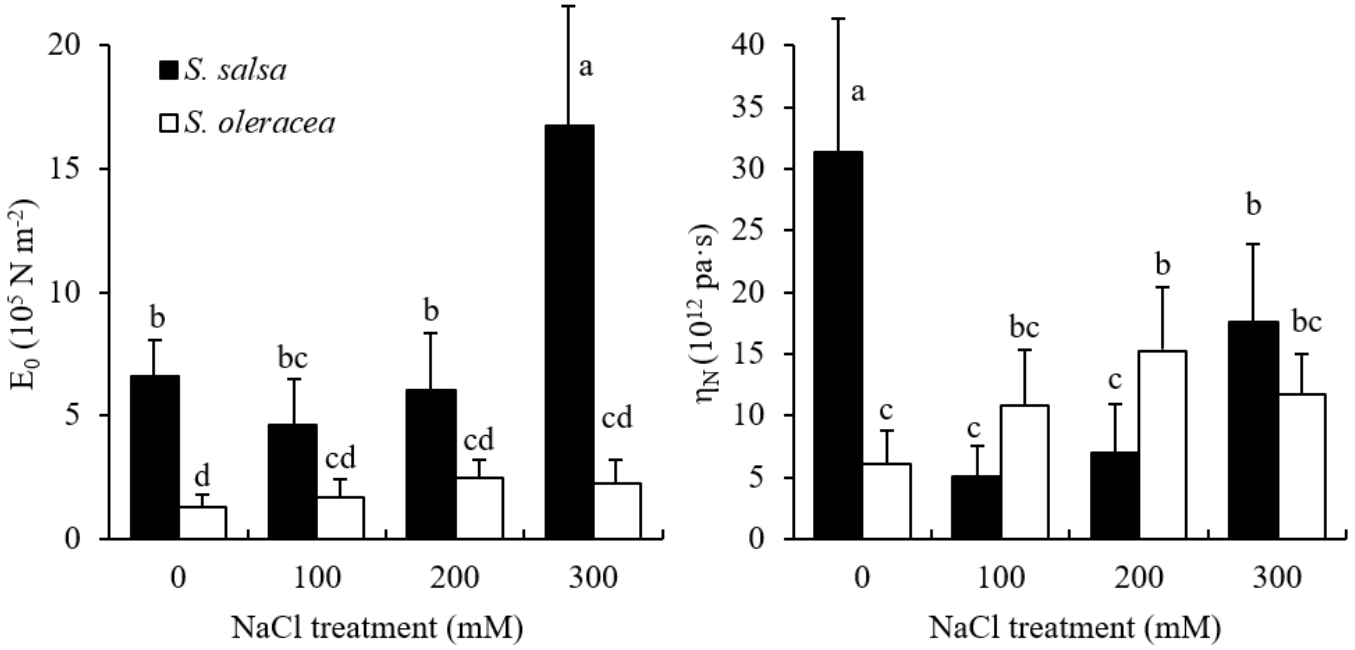

Figure 2-2. Elastic moduli (E_0) and viscosity coefficient (η_N) of the root cell wall in the elongation zone in *S. salsa* and *S. oleracea* in 0, 100, 200, and 300 mM NaCl treatments. Data are mean + S.D. (n = 12−15). Different letters indicate significant differences ($p < 0.05$).

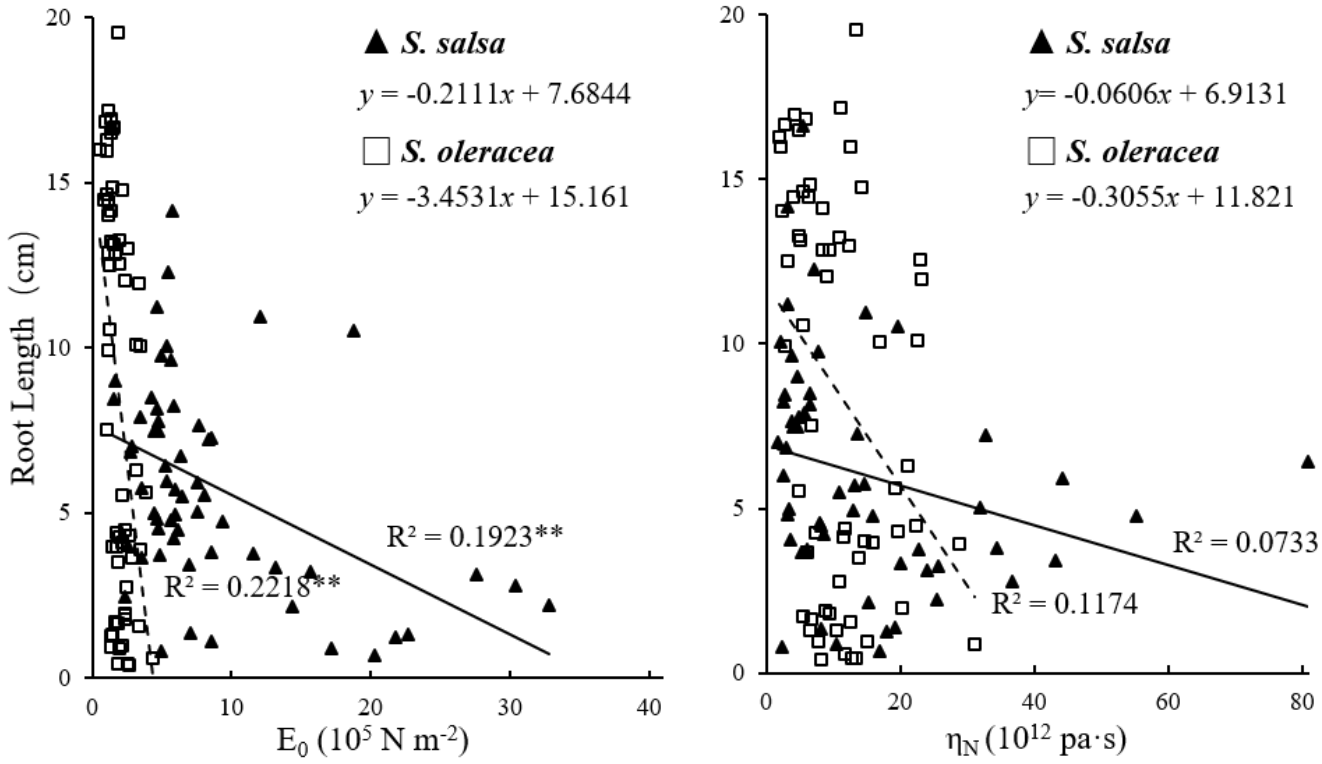

Figure 2-3. Correlation of root length with E_0 (elastic modulus; left panel) and η_N (viscosity coefficient; right panel) in *S. salsa* (—) and *S. oleracea* (- - -) under 0, 100, 200, and 300 mM NaCl treatments. ** significant at $p < 0.01$ ($n = 56-60$).

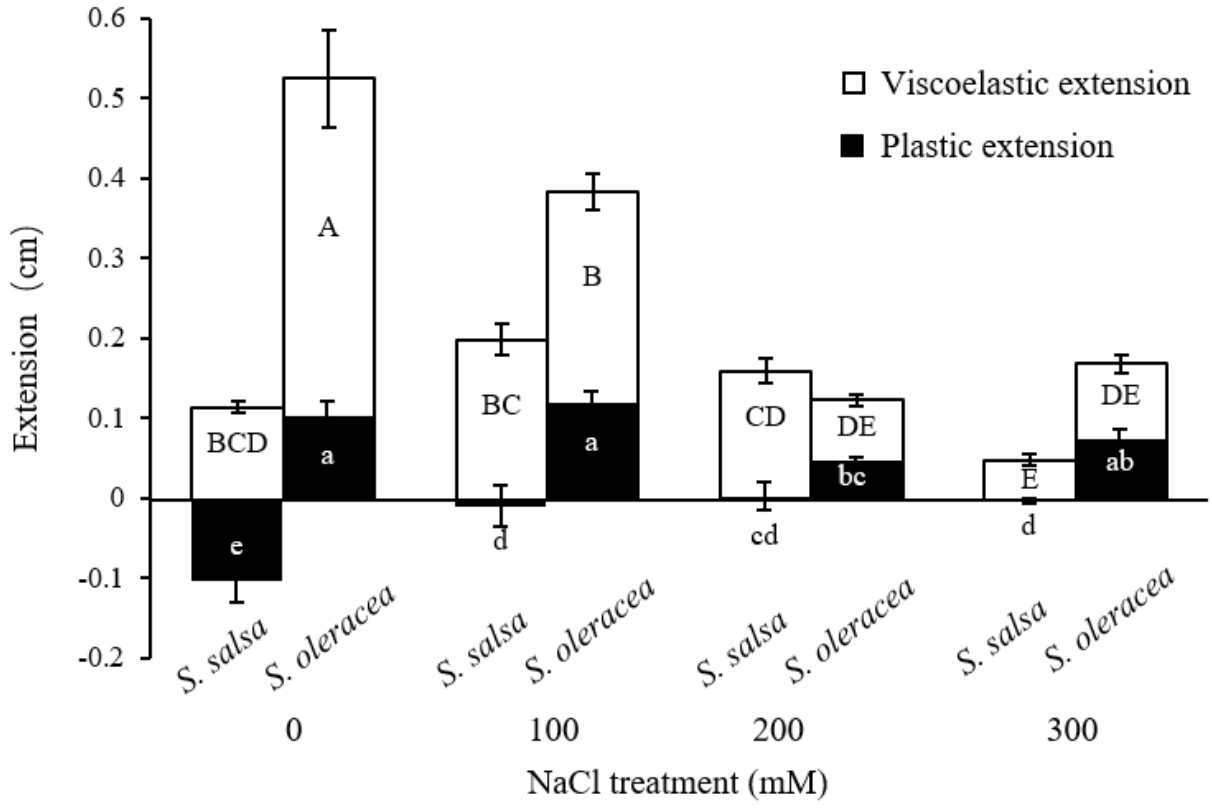

Figure 2-4. Viscoelastic extension and plastic extension in *S. salsa* and *S. oleracea* root grown under 0, 100, 200, and 300 mM NaCl treatments. Data are mean ± S.D. (n = 12−15). Different uppercase (viscoelastic extension) and lowercase (plastic extension) letters indicate significant differences ($p < 0.01$).

2.3.3. Chemical Composition of the Root Cell Wall

Table 2-1 shows the effect of salt treatment on total sugar content in root cell walls. In the 0–5 mm segment behind the root tip, total sugar content was reduced at 100 mM NaCl treatment in both species. The same trend was observed in both 0–5 and 5–10 mm root segments; compared with 100 mM NaCl, 300 mM NaCl significantly increased total sugar content in both species. Moreover, sugar content was nearly two-fold greater in *S. salsa* than in *S. oleracea* across saline treatments. Furthermore, total sugar content was higher in the 5–10 mm root segment than in the 0–5 mm root segment.

Figures 2-5 and 2-6 show the total sugar content in each cell wall fraction in *S. salsa* and *S. oleracea* under different NaCl concentrations. Pectin content decreased in the 0–5 mm region in *S. salsa* root tips at 100 and 300 mM NaCl. Hemicellulose I, hemicellulose II, and cellulose contents initially declined and then increased with increasing saline conditions in *S. salsa* root tips. In contrast, in *S. oleracea*, treatment with NaCl caused a significant decrease in pectin content, coupled with a significant increase in cellulose content at 300 mM NaCl. There was no change in hemicellulose I or hemicellulose II with NaCl treatment (Figure 2-5) in *S. oleracea*. In the 0–5 mm region of the root tips, we observed the same trend in the changes of hemicellulose and cellulose in *S. salsa*, with their content being much higher than those in *S. oleracea*. In the 5–10 mm root segments of *S. salsa*, pectin content decreased at 100 and 300 mM NaCl and cellulose content decreased at 100 mM NaCl (Figure 2-6). Hemicellulose I content decreased in *S. oleracea* at 100 and 200 mM NaCl. Pectin content in the 5–10 mm segments was lower than in the 0–5 mm root segments. Cellulose content in 5–10 mm root segments was higher than in the 0–5 mm root segments across treatments (Figures 2-5 and 2-6). It is worth noting that, except for hemicellulose II, sugar contents of the cell wall fractions in *S. salsa* were higher at 0 mM than at 100 mM NaCl (Figure 2-6).

Table 2-1. Total sugar content in the cell wall of the 0–5 mm and 5–10 mm root segments in *S. salsa* and *S. oleracea* young seedlings grown under different concentrations of NaCl. Data indicate mean ± S.D. (n = 4).

Species	Root Region	Sugar Content (mg g^{-1} FW)			
		NaCl Treatment (mM)			
		0	100	200	300
S. salsa	0–5 mm	43.5 ± 6.4 ab	26.8 ± 7.3 c	37.7 ± 3.7 bc	50.7 ± 3.0 a
S. oleracea	0–5 mm	18.8 ± 2.3 a	15.7 ± 1.1 b	17.4 ± 1.7 a	19.7 ± 1.1 a
S. salsa	5–10 mm	53.9 ± 7.5 ab	30.6 ± 7.9 b	52.9 ± 13.7 ab	61.0 ± 10.3 a
S. oleracea	5–10 mm	21.1 ± 4.5 ab	16.1 ± 2.4 b	19.3 ± 1.4 b	27.0 ± 2.0 a

Different lowercase letters indicate significant difference at $p \leq 0.01$, n = 4. FW, fresh weight.

Uronic acid content in the different cell wall fractions is shown in Figures 2-5 and 2-6. Uronic acid content within the pectin fraction decreased in the 0–5 mm root segments in *S. oleracea* under salinity but remained unchanged except for an increase at 200 mM NaCl in *S. salsa*. An increasing trend in uronic acid proportion in the hemicellulose I, hemicellulose II, and cellulose fractions was observed for both species in response to increasing salt concentration. Uronic acid content in each cell wall fraction generally increased in the 5–10 mm root-tip segments as salt concentration increased from 100 to 300 mM NaCl in both species. In both the 0–5 and 5–10 mm root-tip segments, uronic acid content was significantly higher in the cellulose of *S. salsa* than of *S. oleracea*.

Results of the correlation analysis are shown in Tables 2-2 and 2-3. Hemicellulose I and hemicellulose II contents, including the uronic acid within them, were negatively correlated with root growth in *S. salsa*. In contrast, there was a positive correlation between pectin content and root growth in *S. oleracea*, while cellulose content was negatively correlated with root length in both species. Similarly, there was a positive correlation between the uronic acid content in pectin and root length in *S. oleracea*, but not in *S. salsa*. Furthermore, the uronic acid content in cellulose was negatively correlated to root length in both species. Lastly, pectin content in *S. oleracea* showed significant correlations with root length, cellulose content, and uronic acid content in pectin, hemicellulose II, and cellulose, whereas no such correlations were observed for *S. salsa*.

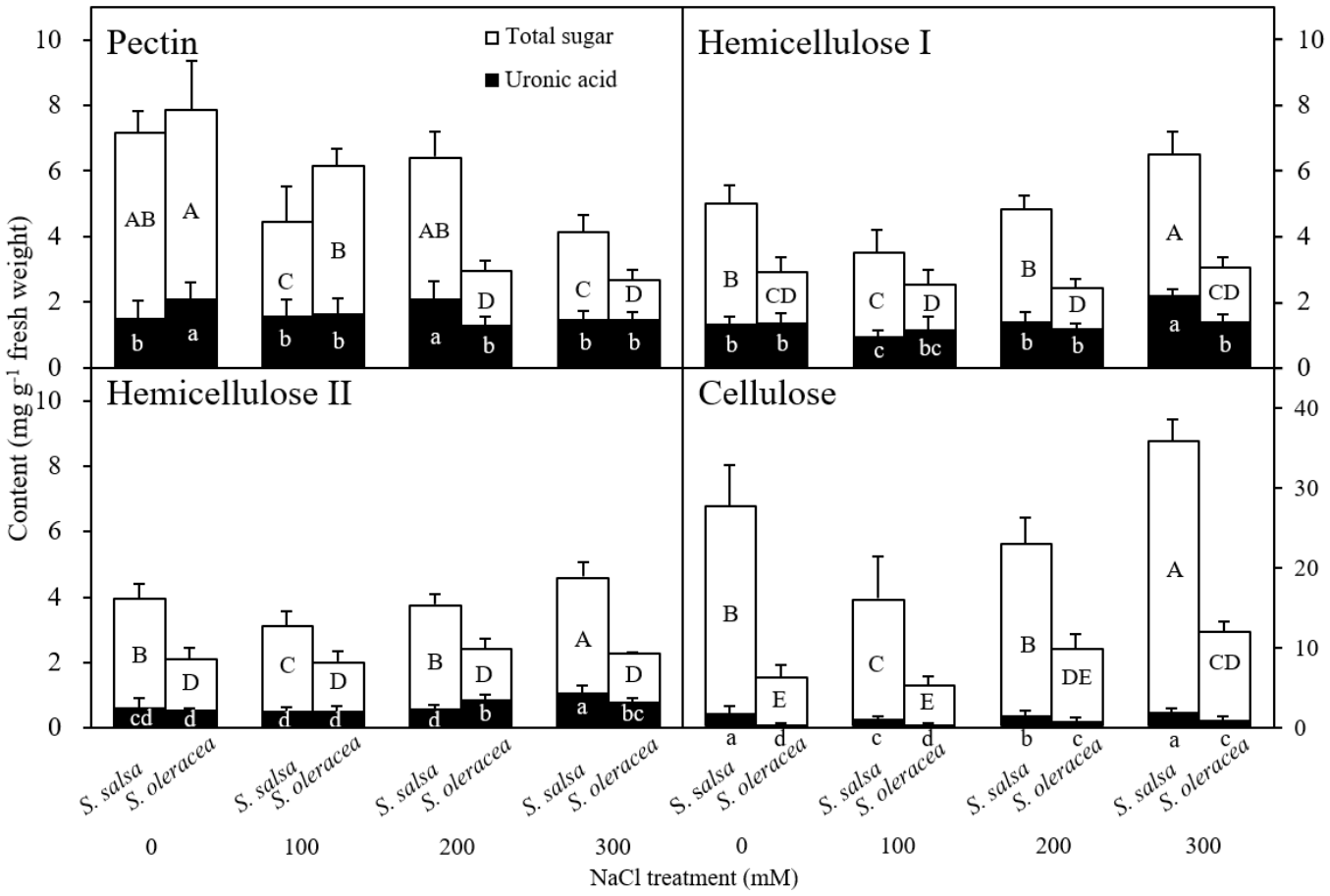

Figure 2-5. Total uronic acid-containing sugar and uronic acid content in the pectin, hemicellulose I, hemicellulose II, and cellulose cell wall fractions in the apical 0−5 mm region in *S. salsa* and *S. oleracea* roots grown under 0, 100, 200, and 300 mM NaCl treatments. Data are mean + S.D. (n = 4). Different uppercase (total sugar) and lowercase (uronic acid) letters indicate significant differences ($p < 0.05$).

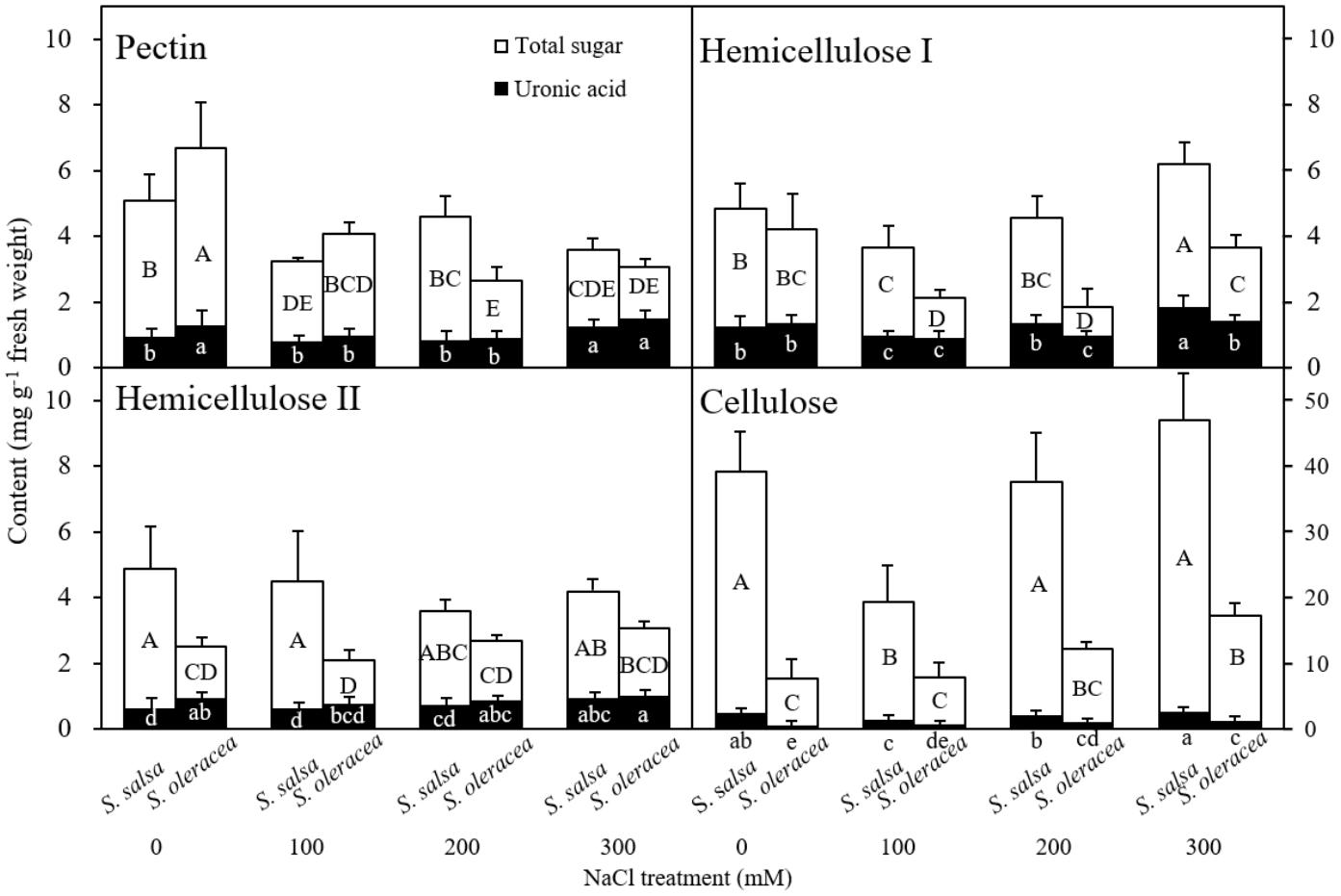

Figure 2-6. Total uronic-acid-containing sugar and uronic acid content in the pectin, hemicellulose I, hemicellulose II, and cellulose cell wall fractions in the apical 5−10 mm region in *S. salsa* and *S. oleracea* roots grown under 0, 100, 200, and 300 mM NaCl treatments. Data are mean + S.D. (n = 4). Different uppercase (total sugar) and lowercase (uronic acid) letters indicate significant differences ($p < 0.05$).

Table 2-2. Cross-correlation coefficients of final root length (RL); total sugar content of pectin, hemicellulose I (HC-I), hemicellulose II (HC-II), and cellulose; uronic acid (UA) content of pectin, HC-I, HC-II, and cellulose in the 0–5 mm region of apical root cap in *S. salsa*.

	RL	Pectin	HC-I	HC-II	Cellulose	Pectin (UA)	HC-I (UA)	HC-II (UA)
Pectin	0.121							
HC-I	−0.868 **	−0.008						
HC-II	−0.814 **	0.005	0.915 **					
Cellulose	−0.882 **	0.04	0.821 **	0.827 **				
Pectin (UA)	0.148	0.287	−0.081	−0.005	−0.003			
HC I (UA)	−0.929 **	−0.268	0.900 **	0.825 **	0.849 **	−0.136		
HC II (UA)	−0.761 **	−0.329	0.766 **	0.739 **	0.811 **	0.048	0.821 **	
Cellulose (UA)	−0.724 **	0.268	0.786 **	0.854 **	0.875 **	−0.016	0.657 **	0.647 **

* Correlation is significant at the 0.05 level (two-tailed); ** Correlation is significant at the 0.01 level (two-tailed). ($N = 14$).

Table 2-3. Cross-correlation coefficients of final root length (RL); total sugar content of pectin, hemicellulose I (HC-I), hemicellulose II (HC-II), and cellulose; uronic acid (UA) content of pectin, HC-I, HC-II, and cellulose in the 0–5 mm region of apical root cap in *S. oleracea*.

	RL	Pectin	HC-I	HC-II	Cellulose	Pectin (UA)	HC-I (UA)	HC-II (UA)
Pectin	0.939 **							
HC-I	−0.055	0.113						
HC-II	−0.417	−0.497	0.276					
Cellulose	−0.826 **	−0.784 **	0.118	0.513 *				
Pectin (UA)	0.718 **	0.851 **	0.459	−0.249	−0.546 *			
HC I (UA)	−0.033	−0.068	0.666 **	0.535 *	0.274	0.214		
HC II (UA)	−0.787 **	−0.845 **	0.117	0.811 **	0.790 **	−0.573 *	0.339	
Cellulose (UA)	−0.884 **	−0.862 **	−0.006	0.576 *	0.958 **	−0.650 **	0.196	0.857 **

* Correlation is significant at the 0.05 level (two-tailed); ** Correlation is significant at the 0.01 level (two-tailed). (N = 14).

2.4. Discussion

A significant negative effect of salinity on root growth was evident even in *S. salsa*, indicating that these plants are sensitive to salinity during early growth. This finding substantiates the use of young seedlings as a suitable experimental material for studies on salt tolerance in plants. Furthermore, the pronounced inhibition in the root growth in *S. oleracea* grown under salt stress showed relatively greater sensitivity to salinity when compared with *S. salsa* (Figures 2-1). Thus, these two related but contrasting species offer a unique opportunity to study the mechanisms underlying species-specific differences in salt-stress response.

Salt stress causes significant cell wall stiffening, which has a detrimental effect on root growth (Zörb et al. 2015). Thus, the changes in root growth may correlate with cell wall extensibility of the root elongation zone. Among the viscoelastic parameters measured in creep-extension, E_0 most represented the overall root extensibility, with greater E_0 values reported for stiffer cell walls, while greater η_N values indicate higher cell wall viscosity (Tanimoto et al. 2000). Thus, the negative correlation observed in this study between E_0 and root growth in *S. salsa* and *S. oleracea* (Figure 2-3) indicates that cell extensibility in the root elongation zone is an important limiting factor of root growth in both halophyte and glycophyte species under saline conditions. Furthermore, the greater value for the regression slope of *S. oleracea* (3.45) than for *S. salsa* (0.21) clearly demonstrates that cell wall stiffness affects root growth more severely in salt-sensitive than in salt-tolerant species via changing extensibility (Figure 2-3). Higher cell wall extensibility (i.e. lower E0) was found to be favorable for root growth under saline conditions. Root cell wall extensibility in *S. salsa* seedlings changed minimally, even when treated with 200 mM NaCl, which would benefit root growth. In contrast, the increased values for E_0 under salt treatment may have resulted in the reduced root growth in *S. oleracea* (Figures 2-1–2-3). The similarity in the viscosity and elasticity of the root cell wall in response to saline conditions suggests that viscosity is also related to root growth (Figures 2-1–2-3). The steeper slopes of the regressions between η_N and root growth in *S. oleracea* than in *S. salsa* indicates that η_N affected root growth in the salt-sensitive species more than in the salt-tolerant species grown in saline conditions

(Figure 2-3). It also shows that extensibility and viscosity are distinctive properties, although they are not theoretically related.

To date, there are no reports of root shrinkage exceeding the original measured root length, which indicates a negative plastic deformation in halophyte roots (Figure 2-4). This phenomenon might be due to the high cellulose content of the cell walls in this species (Figure 2-5). Crystalline cellulose in kraft cooked Norway spruce showed that cellulose chains expand in a direction that is perpendicular to the cellulose chains after a tensile force is applied (Peura et al. 2006). This response of the cellulose chains to tensile force may have caused the vertical shrinkage observed in the *S. salsa* roots. The high viscosity may also have contributed to the shrinkage in the 0 mM NaCl treatment (Figure 2-2). Possible factors contributing to this auxeticity might be the effect of microfibrillar orientation and shearing interactions between neighbor cells and cell wall layers (Park and Cosgrove 2012, Peura et al. 2006). A high amount of cellulose may provide tensile strength and crosslink sites that increase the cell wall stiffness and ensure the cell structure of *S. salsa* under salinity stress. This cell wall property may also limit the elongation of the roots in comparison to S. *oleracea* (Figure 2-1). The correspondence between viscoelastic extension, E_0, and root length (Figures 2-3 and 2-4) shows that cell wall elasticity is an important factor in determining cell elongation and, thus, root length in both halophyte and glycophyte species. Hattori et al. (Hattori et al. 2003) also reported that mechanical cell wall properties determine root growth under drought conditions. Compared to the 0 mM NaCl treatment, less plastic shrinkage in the higher NaCl treatments indicates that salt treatment stiffened the cell walls in *S. salsa* (Figures 2-1, 2-2 and 2-4).

Chemical Composition of the Root Cell Wall

The pectin content in the cell walls of *S. oleracea* roots was consistently affected by salinity (Figure 2-5). These results reveals the sensitivity of pectin to salinity in this glycophyte. Pectin is known to have many important functions in plant meristems, such as ion binding, ion homeostasis, pH adjustment, water retention, and electro-chemical balance (Voragen, Schols and Visser 2013). The Ca^{2+}–pectate and esterification pectin are also critical in controlling the chemical properties and the cell walls' viscoelasticity by affecting cross-linkages to cell wall polymers (Hocq et al. 2017, Peaucelle, Braybrook and Höfte 2012, Peaucelle et al. 2011). These functions

may have been inhibited under saline conditions in *S. oleracea* and resulted in reduced root growth. The reduced pectin content in *S. oleracea* after saline treatment may have reduced cell wall extensibility, which subsequently reduced root growth (Figures 2-1–2-5, Table 2-3).

Cell wall pectin has been found to play a key role in cation binding (Szatanik-Kloc, Szerement and Józefaciuk 2017) because the galacturonic acids in pectin provide cation binding sites (Caffall and Mohnen 2009). The uronic acid content in the pectin fraction of cell walls was significantly correlated with pectin content in *S. oleracea* in response to salinity (Figure 2-5; Table 2-3). Furthermore, the proportion of uronic acid content in pectin increased as salinity increased in both species (Figure 2-5). This finding indicates that uronic acid is an important functional constituent of pectin that plays a significant role in plant tolerance and adaptation to adverse stress conditions. The increased proportion of uronic acid in pectin after NaCl treatment suggests that NaCl enhanced uronic acid synthesis, which may be related to its cation binding ability. Additionally, the lack of glucuronic acid could increase cell wall thickness (Reboul et al. 2011), and may also relate to plant salt tolerance (Byrt et al. 2018). Höfte et al. (Peaucelle et al. 2012) and Le Gall et al. (Le Gall et al. 2015) reported that the degree of pectin methyl esterification was changed by salt stress under salt stress, likely affecting the structural integrity and the mechanical properties of the cell wall. The absolute contents of pectin and uronic acid changed under salinity treatment. Whether these changes affect the degree of pectin methyl esterification is not clear. The interactions of galacturonic acid, pectin methyl esterification and root growth under salinity need to be further investigated. Changes in cell wall extensibility may occur through the synthesis of new cell wall material or changes in cell wall polysaccharides (Ma et al. 2004). We found that NaCl inhibited pectin synthesis while enhancing cellulose synthesis in *S. oleracea*. Thus, pectin content decreased while cellulose content increased, especially at 200 and 300 mM NaCl (Figures 2-5 and 2-6). These findings may explain the decrease in cell wall extensibility observed in the plants grown in the high saline treatment conditions (Figure 2-2). Therefore, NaCl treatment could affect root growth in glycophytes through changes in cell wall synthesis (Figures 2-5 and 2-6, Table 2-1). In contrast, the root growth in halophytes was unrelated to cell wall pectin synthesis.

The primary salinity tolerance mechanism involved in *S. salsa* is Na^+ accumulation and compartmentation in the vacuole (Song et al. 2009). Therefore, the weak correlations between pectin content or uronic acid content (in the pectin fraction) and root growth in *S. salsa* shows that pectin may not be related to salt tolerance in this species (Table 2-2). The significant increase in hemicellulose I, hemicellulose II, and cellulose content with increased concentrations from 100 mM to 300 mM NaCl may have reduced cell wall extensibility, which inhibited root growth in *S. salsa* (Figures 2-1–2-3, and 2-5). Indeed, the fact that both species showed increased cellulose content with increased salt treatments from 100 mM to 300 mM NaCl suggests that cellulose is an important structural constituent, which is affected by salinity in both halophytes and glycophytes (Figures 2-5 and 2-6). Given that increased cellulose synthesis can contribute to the preservation of cell wall integrity and rigidity (Ricardi et al. 2014), the biosynthesis of cellulose in both species probably contributed to the maintenance of cell morphology and assisted in salt stress resistance. In *S. salsa*, changes in pectin, hemicellulose, and cellulose content had the same pattern in all NaCl treatments; however, only pectin showed a difference at 300 mM NaCl (Figure 2-5). *S. salsa* is a halophyte that accumulates biomass even in high saline conditions (Song et al. 2009). The effects of salt stress on cell wall synthesis and damage to pectin- and cellulose-related components can alter salt tolerance (Kesten, Menna and Sánchez-Rodríguez 2017, Mustard and Renault 2004). We speculate that, due to its specific salt-tolerance mechanisms and because it contains almost twice as much hemicellulose and cellulose as *S. oleracea*, *S. salsa* has a more stable cell wall architecture under salt stress conditions (Table 2-1; Figure 2-5). A highly saline condition disrupts cross-linking between pectin and other cell wall fractions (Feng et al. 2018), which affects the stability of the cell wall and significantly inhibits root growth. A stable cell wall architecture may contribute to the overall salt tolerance of *S. salsa*.

Our study shows that, for two contrasting members of the Amaranthaceae, the halophyte *S. salsa* and glycophyte *S. oleracea*, the effects of salinity stress on root growth are closely related to the mechanical properties and chemical composition of the cell wall. Salinity affects root growth through the processes of cell wall loosening and synthesis. Cellulose may provide mechanical strength, but it limits root elongation under saline conditions. In halophytes, the high content of the cell wall and

the proportion of cellulose in the cell wall may be a salt tolerance mechanism that protects the cell structure's stability under salt stress. Pectin played important roles in both the glycophyte and halophyte, but the effect was more pronounced in the glycophyte, possibly because of their different salt tolerance mechanisms. The glycophyte relies on salt exclusion, whereas the halophyte relies on salt absorption and compartmentation in vacuoles. The function of the root cell wall in root growth was more prominent in the glycophyte than in the halophyte under saline conditions.

Chapter 3 Pectin Characteristics Affecting Root Growth in Spinach under Salinity

3.1. Introduction

Soil salinity is an important environmental problem for more than 800 million hectares of land, which results in osmotic stress, ionic imbalances, ion toxicity, oxidative damage and complex effects on the physiology and metabolism of plants, including spinach (*Spinacia oleracea* L.) (Negrão, Schmöckel and Tester 2017, Ors and Suarez 2016). The growth of most spinach crops are reduced when the soil salinity exceeds 4 dS/m of electrical conductivity, which is equivalent to 40 mM sodium chloride (Scudiero et al. 2017, Munns et al. 2020). Although excessive salts are toxic to salt-sensitive plants, some cultivars in spinach may be salt tolerant once adapted to a moderate saline stress. Exposure to high salt concentration adversely affects crop performance due to salinity-induced nutritional imbalance. Studying the physiological responses of different salt tolerance cultivars to salinity in spinach would provide a useful tool for understanding the mechanisms underlying plant responses to salinity.

Root cell wall is a major storage site for several environmental polluttants, including salinity. It acts as a protective barrier of protoplasts by trapping toxic substances to reduce cellular damages mainly caused by salts, or other trace metals (Richter et al. 2017). Its important role in plant resistant to salinity stress could be attributed to the interaction with salts present in plants and soil solution (An et al. 2014, Shao et al. 2021a, Tenhaken 2015). The cell wall matrix is composed of pectin, hemicellulose, and cellulose. Pectin is composed of homogalacturonan (HG) and rhamnogalacturonan I (RG-I) (Albersheim et al. 1996), the main changes in cell wall following salinity stress were found in the pectin sugar composition, pectin characteristics such as HG:RG-I and degree of methyl esterification (Corrêa-Ferreira et al. 2019, Huang et al. 2017, Huang et al. 2016). The synthesis of galactose and arabinose side-chains are also considered to contribute to maintaining cell wall integrity under salinity stress (Corrêa-Ferreira et al. 2019, Zhao et al. 2020).

Plant cell wall is essential for strength, growth and development of plants (Caffall and Mohnen 2009). Cell walls of spinach contain phenolic acids (ferulic, p-coumarics, and diferulic) which are bound to polysaccharide compounds, including pectin. These affect the physical properties of cell walls by increasing the calcium cross-links between homogalacturonans, resulting in stiffening of the pectin gel and primary cell walls (Cosgrove 2016). Xiong et al. (Xiong et al. 2015) reported that cell expansion in rice seedlings cultured in the absence of Ca^{2+} was still regulated by pectin. Huang et al. (Huang et al. 2017) reported that a modified pectin structure can provide different strength for cell wall architecture. However, there are few studies on the structural changes of cell wall pectin under salinity stress. Our previous research revealed that cell wall pectin played important roles in cell wall extension in both *Spinacia oleracea* and *Suaeda salsa* under salinity, and that the salt tolerance of *S. oleracea* was affected by pectin (Liu et al. 2022).

Spinach (*Spinacia oleracea* L.), being a well-known leafy vegetable with various salinity tolerance levels in different cultivars has been recently reported (Liu et al. 2022, Kim et al. 2021, Ors and Suarez 2016, Turhan, Kuşçu and Şeniz 2011, Xu and Mou 2016). In this study, we investigated the salinity tolerance of three spinach cultivars with focus on pectin content such as: pectin polysaccharides, pectin methylesterification degree (PMD) and pectin related wall parameters in the cell walls.

3.2. Materials and methods

3.2.1. Plant materials

The seeds of *Spinacia oleracea* L., salt sensitive-'Helan 3' (bred in Holland) and 'Prius β' Denmark), and salt tolerant-'R7' (bred in Japan) were purchased from a seed market in the city of Tottori, Japan. The evaluation of their salt tolerance was based on effects of salinity stress on root length, which is an important indicator for evaluating salt tolerance (Xu and Shi 2007, Demiral and Türkan 2005). Seeds of Spinach were washed and soaked in distilled water for 24 h. Seed germination and seedling growth were conducted in growth chambers (MLR-350HT; Sanyo, Osaka, Japan) at 20 °C. Fifteen seeds were aligned on a sheet of filter paper in a zip-lock plastic bag. Filter papers were moistened every day during the three-day germination period for Spinach in dark. After germination, 1/12 diluted Hoagland solution,

containing 0 and 200 mM NaCl treatments, was applied to the roots every 2 days (d). Seedlings were subjected to salinity treatments for 6 d. Light conditions were set to 12/12 h cycles (day/night). Each salt concentration treatment comprised of 24 filter paper germination sheets. At the end of germination test, six filter papers were randomly selected from each treatment combinations for the measurement of root length of each seedling.

3.2.2. Mechanical parameters of the root cell wall

Root samples separated from cultured seedlings were excised 10 mm from the apical zone and immediately transferred to boiling methanol in a water bath (80 °C, 5 min). Methanol-killed root segments were rehydrated with 1/12 diluted Hoagland solution (pH 6.5) and extended. We determined the root extensibility following Tanimoto et al. (Tanimoto et al. 2000). The cell wall extensibility and viscosity were measured using a creep meter (RE2-33005C-1,2; Yamaden, Tokyo, Japan). A 3-7 mm root segment behind the root cap was fixed between the two clamps of the creep meter used for the measurement of extension. Roots were stretched under 0.1 N tensile force for 5 min and then released for 5 min. The final length at 5 min was read as reversible extension (elastic extension), while the length difference between final length, and original length (4 mm) was read as plastic extension. The elastic modulus (E_0) and the viscosity coefficient (η_N) were determined using the software supplied with the creep meter, which indicated the extensibility and viscosity, respectively (Tanimoto et al. 2000). An increase in E_0 value indicates a decrease in elasticity, while greater η_N values indicated higher cell wall viscosity (Tanimoto et al. 2000). We measured the cell wall physical parameters of at least 18-25 root segments for each replicate.

3.2.3. Extraction of cell wall fractions

Seedling roots were taken out from growth bags, thoroughly washed with distilled water and cut into 10 mm segments behind the root tips as elongation zones (Nonami et al. 1994). About 50 segments from 4 filter paper sheets were taken as one replicate, while 6 replicates were measured for one treatment.

Cell wall pectin was extracted using the procedure in An et al. (2014). The root segments were immediately homogenised in a mixture of ice-cold Tris-HCl buffer

(pH 7.4) and Tris buffer-saturated phenol using a bead crusher (Model μT-12; TAITEC Co. Ltd. Tokyo, Japan). The homogenate was centrifuged at 3800 g for 10 min at 10 °C. The supernatant was discarded and the pellet containing the cell walls was further purified by sequential incubation and centrifugation in ethanol, acetone, a mixture of methanol: chloroform (1:1, v/v), and again in acetone and ethanol. The centrifuged residues were designated as cell walls after treated with pronase in phosphate buffer (pH 7.0). The pectin fractions were extracted five times with CDTA at pH 6.5 at 20 °C. To extract the remaining polyuronides, cell walls were further extracted three times with CDTA at 100 °C (hot CDTA) for 1 h each. These CDTA extractions were designated as the pectin fraction.

3.2.4. Characterisation of the extracts

3.2.4.1. Sugar composition

The amounts of total sugars and uronic acid in each extract of cell wall were measured using the phenol-sulfuric acid method (Dubois et al. 1951) and *m*-hydroxydiphenyl colorimetric method (Ahmed and Labavitch 1978), respectively.

Pectin fraction was hydrolyzed with 4 M trifluoroacetic acid at 100 °C for 6 h in a sealed tube. Excess trifluoroacetic acid was removed by evaporation under reduced pressure. Neutral monosaccharides (Rhamnose, Arabinose, Xylose, Mannose, Glucose and Galactose) in pectin were derivatised and analysed as their acetylated derivatives using gas chromatography (GC) (Zhao et al. 2014). Pectin fraction was hydrolysed with 4 M trifluoroacetic acid at 100 °C for 6 h in a sealed tube. Trifluoroacetic acid was removed by evaporation under reduced pressure (Speedvac SPD131DDA, Thermo Scientific, Waltham, MA, USA), 5 mg ammonium hydrochloride and 0.5 mL pyridine were added and allowed to react in a 90 °C water bath for 30 min. Acetic anhydride (0.5 mL) was added to the test tube and incubated at 90 °C for another 30 min to allow the acetylation reaction to occur. The acetylated derivatives were analyzed by GC (GCMS-QP2010C Plus, SHIMADZU, Kyoto, Japan) with a HP-5MS column (0.25 mm × 30 m × 0.25 μm) and a flame-ionization detector. The temperature programme was set at 130 °C and maintained for 5 min and then increased to 240 °C at an increment of 5 °C/min. The 240 °C temperature was held for 5 min. The HG:RG-I ratio was calculated by GalA / Rha, side-chain length of

galactan and arabinogalactan were Gal / Rha and (Ara + Gal) / Rha in mol%, respectively (Huang et al. 2017).

3.2.4.2. Determination of pectin methyl-esterification

The pectin methyl-esterification degree (PMD) was quantified by the amount of methanol produced using enzymatic pectin hydrolysis and colorimetric method as described in Anthon et al. (Anthon and Barrett 2008). Pectin samples were mixed with alcohol oxidase 30 °C in a water bath. After 10 mins, freshly prepared 5 mg/mL Purpald in 0.5M NaOH was added, and the mixture incubated for an additional 40 min at 30 °C. Methanol content was determined at 550 nm absorbance using UV-visible spectrophotometer (Shimadzu UV−1900i, Shimadzu, Tokyo, Japan). The PMD was calculated as the moles of methyl esters groups per 100 mol of uronic acid.

3.2.5. Statistical Analysis

All data were analyzed using the analysis of variance (ANOVA) and correlation; means were compared using Tukey's Honestly Significant Difference test ($P < 0.05$). All statistical analyses were performed using SPSS software version 28.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.3.1. Root growth

Salinity significantly inhibited root elongation in all Spinach cultivars (Figure 3-1A). Root growth across the cultivars was significantly inhibited under 200 mM NaCl treatment. This inhibition was more pronounced in Helan 3 and Prius β, compared with R7, which was a more tolerant cultivar. The diameter of the roots of all three cultivars increased significantly under salinity (Figure 3-1B). There was a 44 and 46 % increase in rooting diameter in Helan 3 and R7 under salinity stress, respectively whereas, 13 % increase was observed in Prius β (Figure 3-1B). Pectin methyl-esterification degree (PMD) had a significant ($P < 0.05$) positive correlation with root length in R7 (Table 3-3).

3.3.2. Root cell wall extensibility and viscosity

The elastic moduli of E_0 in root elongation zone in all three cultivars increased significantly under salinity stress (Figure 3-2). The E_0 in salt sensitive cultivar Helan 3 was significantly higher compared with the salt tolerant cultivar R7, whether under 0 or 200 mM NaCl. The viscosity coefficient, η_N, was significantly increased in sensitive cultivar Helan 3 and tolerant cultivar R7. However, there was no significant change in Prius β (Figure 3-2). Meanwhile, in 0 mM NaCl treatment, the viscosity coefficient of Prius β was significantly higher than those of the other two cultivars (Figure 3-2). Pectin methyl-esterification degree (PMD) had a negative significant ($P < 0.05$) relationship with E_0 and η_N in R7, relative to the other two cultivars. (Table 3-3).

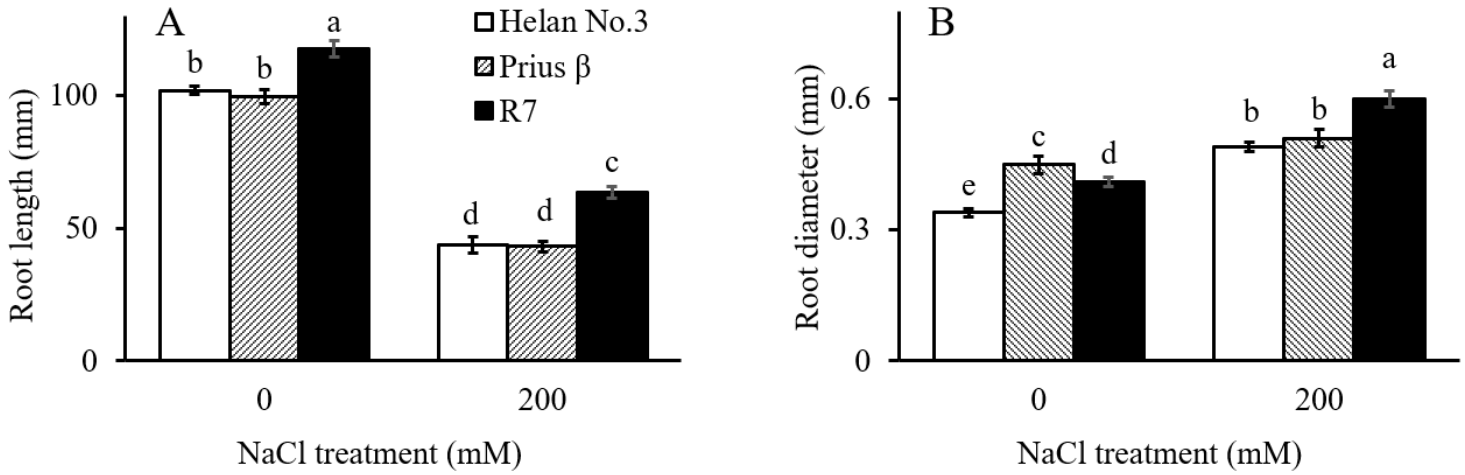

Figure 3-1. Final root length, relative root length (A) and root diameter (B) of Helan 3, Prius β and R7 in 0 and 200 NaCl treatments. Data are mean ± S.E. (n = 6). Different letters indicate significant differences ($P < 0.05$).

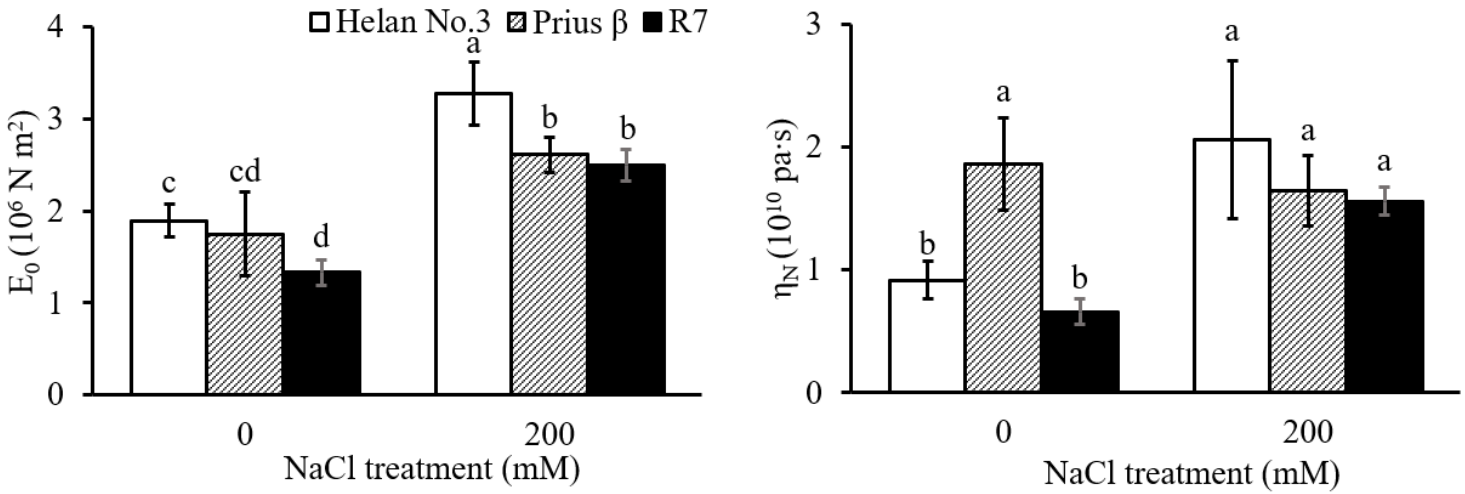

Figure 3-2. Elastic moduli (E_0) and viscosity coefficient (η_N) of the root cell wall in the elongation zone in Helan 3, Prius β and R7 in 0 and 200 mM NaCl treatments. Data are mean ± S.E. (n = 15-24). Different letters indicate significant differences ($P < 0.05$).

3.3.3. Chemical composition of root cell wall

Salinity treatment significantly increased pectin content of root cell wall in all cultivars (Figure 3-3). The molar proportion of each monosaccharide component in the pectin across the cultivars are shown in Table 3-1. Salinity increased the molar proportion of rhamnose, arabinose and galactose in the pectin of Helan 3 and R7, while uronic acid molar proportion in the cultivars decreased (Table 3-1). In Prius β cultivar, the molar proportion monosaccharide compositions had no significant changes in all monosaccharide components under salinity stress (Table 3-1).

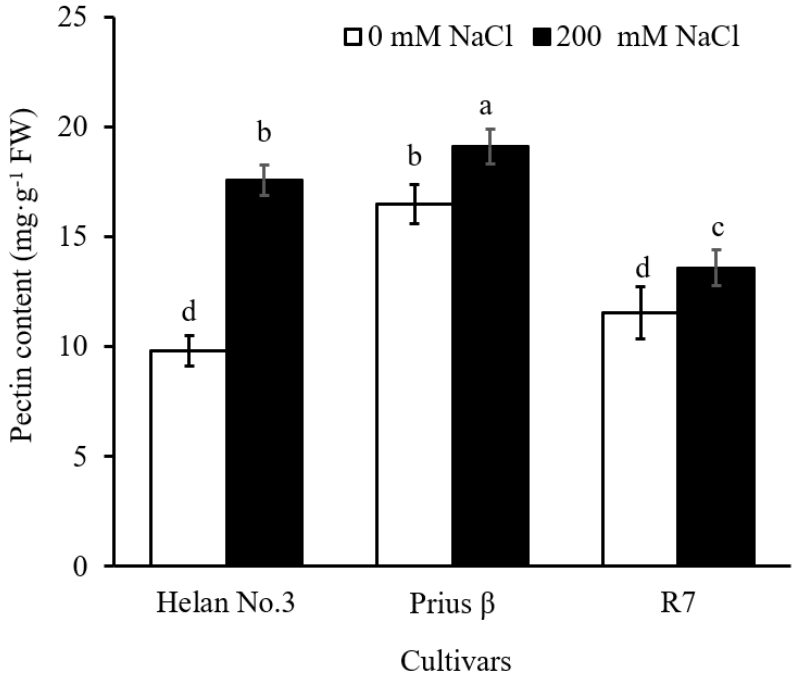

Figure 3-3. Pectin contents in root cell wall in three cultivars, Helan 3, Prius β and R7 under 0 and 200 mM NaCl treatments. Data are mean ± S.E. (n = 6). Different letters indicate significant differences ($P < 0.05$).

Table 3-1. Monosaccharide composition (mol %) of rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), galactose (Gal) and uronic acid (UA) in Helan 3, Prius β and R7 cultivars under salinity stress. Data are mean ± S.E. (n = 6).

Cultivar	NaCl (mM)	Mol %						
		Rha	Ara	Xyl	Man	Glc	Gal	UA
Helan 3	0	5.9 (0.4) c	9.2 (0.6) b	4.3 (0.4) a	2.4 (0.2) b	8.1 (1.3) a	14.7 (0.8) c	55.5 (2.1) a
	200	7.5 (0.3) b	12.1 (0.7) a	5.1 (0.5) a	4.0 (0.6) b	5.1 (1.1) ab	17.9(1.2) ab	48.3 (2.3) b
Prius β	0	6.2 (0.7) c	11.1(1.3) ab	3.1 (0.5) b	9.7 (1.2) a	2.7 (0.5) b	19.8 (2.1) a	47.4 (3.9) b
	200	6.2 (0.4) c	9.8 (0.6) ab	2.1 (0.2) bc	11.2 (0.9) a	1.7(0.2) b	17.6(1.2) ab	51.4(3.1) ab
R7	0	8.8 (0.8) b	10.6 (0.9) b	2.4 (0.3) bc	1.3 (0.1) b	4.2 (0.5) b	14.6 (1.0) c	58.2 (2.6) a
	200	10.4 (0.7) a	12.5 (0.8) a	1.5 (0.3) c	2.5 (0.2) b	5.1 (0.7) ab	20.9 (1.3) a	47.0 (2.9) b

Means followed by the same letter in the same column are not significantly different ($P < 0.05$).

Table 3-2 shows the effect of salt treatment on pectin characteristics including pectin methyl-esterification degree (PMD) in pectin fractions, HG:RG-I ratio, galactan side-chain length and arabinangalactan side-chain length. In R7 cultivar, the PMD decreased significantly and the length of galactan and arabinangalactan side-chains significantly increased when exposed to salinity, while there were no significant changes in Helan 3 and Prius β cultivars (Table 3-2). HG:RG-I ratio were significantly decreased in Helan 3 and R7 cultivars, with no significant change in Prius β cultivar (Table 3-2).

Root growth was negatively correlated with pectin content, E_0 and root diameter across the cultivars (Table 3-3). Both E_0 and η_N in Helan 3 and R7 were significantly correlated positively and negatively with pectin content and uronic acid molar proportion of pectin, respectively (Table 3-3).

Table 3-2. Pectin methyl-esterification degree (PMD) in pectin fractions, HG:RG-I ratio, galactan side-chain length and arabinangalactan side-chain length in Helan 3, Prius β and R7 cultivars under salinity stress. Data are mean ± S.E. (n = 6).

Cultivar	NaCl (mM)	PMD (%)	HG:RG-I ratio	Galactan side-chain	Arabinangalactan side-chain
Helan 3	0	35.1 (6.7) c	9.8 (1.1) a	2.6 (0.3) ab	4.2 (0.3) b
	200	31.4 (2.8) c	6.5(0.7) bc	2.4 (0.1) bc	4.0 (0.1) bc
Prius β	0	42.4 (3.5) b	8.3 (1.5) ab	3.3 (0.3) a	5.0 (0.3) a
	200	38.8 (3.2) bc	8.6 (1.1) ab	2.8 (0.1) ab	4.4 (0.1) ab
R7	0	59.0 (6.9) a	7.0 (0.8) ab	1.7 (0.1) d	2.9 (0.1) d
	200	42.5 (2.2) b	4.7 (0.6) c	2.0 (0.1) c	3.2 (0.1) c

Means followed by the same letter in the same column are not significantly different ($P < 0.05$).

Table 3-3. Cross-correlation coefficients of final root length, elastic moduli (E_0), viscosity coefficient (η_N) pectin content in cell wall, uronic acid molar proportion of pectin, HG:RG-I ratio and pectin methyl-esterification degree (PMD) of root cell wall in spinach under salinity stress.

	Root length	E_0	η_N	Pectin content	Uronic acid	HG:RG-I ratio	PMD
Helan 3							
E_0	-0.875**						
η_N	-0.868**	0.780**					
Pectin content	-0.885**	0.685*	0.825**				
Uronic acid	0.601*	-0.656*	-0.623*	-0.268			
HG:RG-I ratio	0.621*	-0.556	-0.638*	-0.640*	0.325		
PMD	0.247	0.006	-0.355	-0.395	0.063	0.352	
Root diameter	-0.889**	0.591*	0.740**	0.927**	-0.344	-0.579*	-0.412
Prius β							
E_0	-0.628*						
η_N	0.275	-0.264					
Pectin content	-0.712**	0.384	0.180				
Uronic acid	-0.175	0.114	-0.869**	-0.291			
HG:RG-I ratio	0.013	0.098	-0.838**	-0.408	0.964**		
PMD	0.227	0.057	-0.185	-0.235	0.056	0.056	
Root diameter	-0.659*	0.286	-0.611*	0.586*	0.363	0.363	-0.086
R7							
E_0	-0.816**						
η_N	-0.821**	0.879**					
Pectin content	-0.737**	0.689*	0.741**				
Uronic acid	0.708**	-0.611*	-0.666*	-0.557			
HG:RG-I ratio	0.615*	-0.633*	-0.657*	-0.512	0.979**		
PMD	0.807**	-0.657*	-0.651*	-0.581*	0.450	0.351	
Root diameter	-0.914**	0.779**	0.786**	0.823**	-0.656*	-0.585*	-0.687*

*Correlation is significant at the 0.05 level (two-tailed); ** Correlation is significant at the 0.01 level (two-tailed). (N = 12).

3,4. Discussion

3.4.1. Root growth

Spinach belongs to a broad family of Amaranthaceae, which shows relatively high salt tolerance. But due to different cultivars selected, differences in salt tolerance among cultivars were also reported (Liu et al. 2022, Kim et al. 2021, Turhan et al. 2011, Xu and Mou 2016). Root growth of Helan 3 and Prius β were more affected by salinity, but R7 showed higher root growth (Rozema and Schat 2013). This indicates that R7 cultivar had higher salt tolerance than the other two cultivars. Root growth could be affected by various factors under salinity. Pectin is one of these factors which has been reported to regulate root growth (Peaucelle et al. 2011). The polysaccharides, degree of esterification, HG:RG-I ratio and neutral side-chains of pectin may all affect cell elongation and root growth because these pectin constituents affect cation binding, pH adjustment and ion homeostasis in cell wall (Peaucelle et al. 2011, Feng et al. 2018, Liu et al. 2022, Rozema and Schat 2013). Under salinity stress, all cultivars showed siginificant increase in root diameter. This may be due to apoplast pH decrease (Jaskowiak et al. 2019, Shao et al. 2021b). In barley, a decrease in pH resulted in an increase in rhizodermal cell diameter (Jaskowiak et al. 2019), thereby thickening the roots. Our correlation analysis is consistent with these results (Table 3-3).

3.4.2. Root cell wall extensibility and viscosity

Cell wall extensibility is known to regulate cell elongation. This extensibility is associated with cell wall structure and composition (Neumann et al. 1994, Munns 2002). It was recently reported that salinity stress could alter cell wall structure and composition, thereby, affecting wall extensibility (Byrt et al. 2018). Higher cell wall extensibility is favorable for root growth under saline conditions (Tanimoto et al. 2000). In this study, the low E_0 (i.e., high extensibility) in R7 compared with Helan 3 may have contributed to its higher root growth under salinity. The negative correlation between E_0 and root length across the cultivars indicates that cell wall extensibility in root elongation zone is important for root growth under saline condition (Figure 3-2, Table 3-3).

Under salinity stress, cell volume shrinkage and cell wall deformity occurs through increased cell wall synthesis and strength (Liu et al. 2022, Hu et al. 2019, Novaković et al. 2018, Taiz 1984). Reboul et al. (Reboul et al. 2011) reported that the lack of glucuronic acid could limit cell wall expansion. Similarly, Zdunek et al. (Zdunek et al. 2016) reported that the amount of uronic acid in cell walls may be related to its stiffness, especially in pear plant. In this study, pectin content, uronic acid molar proportion in pectin and HG:RG-I ratio were significantly correlated to E_0 under salinity stress in Helan 3 and R7 cultivars. These correlations indicates that cell wall extensibility was affected by pectin in Helan 3 and R7 cultivars, which may have affected cell expansion under the stress condition. Moelants et al. (Moelants et al. 2013) reported that pectin viscosity was affected by polysaccharide chain structure in carrot and tomato. This is also in line with the report of Mierczyńska et al. (Mierczyńska et al. 2015) that pectin viscosity is related to uronic acid content, and a smaller pectin molecule could lead to increased viscosity in carrot. Also, Pieczywek et al. (Pieczywek et al. 2017) reported an increase in GalA (galacturonic acid) content that led to softening of cell walls. The uronic acid molar proportion in pectin and HG:RG-I ratio were significantly correlated with η_N across the cultivars, this is an indication that uronic acid in pectin and RG-I backbone may have regulated cell wall viscosity during salinity stress in spinach.

3.4.3. Chemical composition of root cell wall

Pectin content significantly increased across the cultivars under salinity stress (Figure 3-3). Whereas the molar proportion of uronic acid in pectin was significantly decreased in Helan 3 and R7 cultivars (Table 3-1). There was a positive correlation between the molar proportion of uronic acid in pectin and root length of the two cultivars (Table 3-3). This indicates that pectic uronic acid was consistent with root growth in the two cultivars. A previous study reported that uronic acids in pectin had been found to provide cation binding sites, which can trap Na^+ to reduce cellular damages (Caffall and Mohnen 2009, Richter et al. 2017). Interestingly, combined Na^+ and uronic acid was found to release H^+ which adjusted the pH of apoplast and consequently altered the expansin, thereby affecting cell wall extensibility, and thus improved root growth (Feng et al. 2018, Shao et al. 2021b). In this study, no significant effect of uronic acid on root growth was found in all the three cultivars. A

similar trend was observed in *Suaeda salsa* and *S. oleracea* 'Akinokagayaku' (Liu et al. 2022), which belonged to the same family of Amaranthaceae. The role of uronic acid under salinity stress may be species-dependent.

In R7 cultivar, PMD decreased significantly but did not show any significant difference in the other two cultivars. The PMD was significantly correlated to root length, pectin content, E_0 and η_N in R7, relative to the other cultivars. This is indicative that salt tolerance in plant cultivars could be regulated by demethylation of pectin. Zheng et al. (Zheng et al. 2020) and John et al. (John et al. 2019) disclosed that Na^+ induced de-esterification of pectin could result in the formation of an egg-box structure with divalent cations in the form of a gel, and the concentration of sodium ions will affect the crosslinking strength. The increased galactan and arabinangalactan side-chains under salinity stress in R7 may improve network formation. Pectin gels can be embedded in cellulose-hemicellulose networks and contribute to cell wall elasticity (Cosgrove 2016), which may possibly be achieved through the amount of pectin gel on cell wall hydration and demethylation of pectin (Cosgrove 2016, Kennedy et al. 2007, Kirui et al. 2021, White et al. 2014). PMD determines negative charges and has a close negative correlation with ion adsorption in cell walls of plant roots (Eticha, Staß and Horst 2005, Li et al. 2019). The increases in cell wall elasticity also correlated with PMD (Peaucelle et al. 2011). The positive correlation between PMD and root length in R7 indicated that the extensive demethylation of pectin enhanced salt tolerance, and the correlation in PMD and E_0 in R7 cultivar could be attributed to the decrease in PMD that correlated negatively with the cell wall extensibility. The increased side-chains and demethylation of pectin under salinity stress may be the reason for the higher salt tolerance of R7 other than Prius β and Helan 3.

Uronic acid molar proportion of pectin and HG:RG-I ratio were found to be significantly correlated to cell viscosity. RG-I backbone are reportedly involved in the regulation of the water-binding capacity of potato cell walls (Kesten et al. 2017). The viscosity of cell walls in apple plants was also reported to affect its water-binding capacity (Vetter, Kunzek and Senge 2001). This cell wall viscosity could be regulated by the water-binding capacity provided by pectin characteristics (HG:RG-I ratio). In Broxterman and Schols (Broxterman and Schols 2018), pectin and cellulose were reported to have been linked by short and highly branched galactose and arabinose

side-chains on the RG-I backbone. Therefore, salinity stress had increased the length of galactose and arabinose side-chains in R7 (Table 3-2), while they were decreased substantially in Helan 3 and Prius β cultivars (Table 3-2). The increased length of galactose and arabinose side-chains under salinity stress may provide more binding sites for pectin and cellulose, thereby increasing cell wall stability. This may possibly benefit the salt tolerance in R7.

In conclusion, cell wall pectin played important roles in regulating root growth and root diameter under salinity stress. Pectin can affect cell wall viscosity, which may be related to the uronic acid molar proportion or the HG:RG-I ratio in spinach cultivars. In comparing Helan 3 and Prius β cultivars, the high salt tolerance of R7 cultivar was significantly correlated with the pectin characteristics. The demethylation and increased side-chains of pectin under salinity stress may lead to changes in cell wall elongation, and thus root growth, which fundamentally enhance plant growth under salt tolerance.

Chapter 4 General Conclusions

In this study, we investigated cell wall composition, extensibility, and viscosity in the root elongation zone of young seedlings of halophyte (*Suaeda salsa*) and glycophyte (*Spinach oleracea* cv. 'Akinokagayaku'), which both belong to the Amaranthaceae family. We further investigated the salinity tolerance of three spinach cultivars (Helan 3, Prius β and R7) focusing on pectin content, such as: pectin polysaccharides, the degree of pectin methy-lesterification (PMD) and pectin-related wall parameters in the cell walls. The objectives of this study were elucidating the interactions of cell wall composition, extensibility and root growth under salinity stress and pectin characteristics of root cell wall that contribute to root growth under salinity. The main conclusion are described as follows:

(1) For two contrasting members of the Amaranthaceae, the halophyte *S. salsa* and glycophyte *S. oleracea*, the effects of salinity stress on root growth are closely related to the mechanical properties and chemical composition of the cell wall. Salinity affects root growth through the processes of cell wall loosening and synthesis. Cell wall pectin plays important roles in cell wall extension in both species under salinity, and that the salt tolerance of glycophyte *S. oleracea* is affected by the pectin. Cellulose limits root elongation under saline conditions in both species, but in halophytes, a high cell wall content and the proportion of cellulose in cell walls may be a salt tolerance mechanism that protects the stability of cell structure under salt stress. The role of the cell wall in root growth under salinity is more prominent in the glycophyte than in the halophyte.

(2) The cell wall pectin played important roles in regulating root growth and root diameter in spinach cultivars under salinity stress. Pectin can affect cell wall viscosity, which may be related to the molar proportion of uronic acid or the HG:RG-I ratio in spinach cultivars. Compared with Helan 3 and Prius β cultivars, the high salt tolerance of the R7 cultivar was significantly correlated with the pectin characteristics. The length and degree of pectin methy-lesterification of neutral side chains were significantly decreased in the R7 cultivar, with no significant changes in the other two cultivars. The demethylation and increased side chains of pectin under salinity stress may lead to changes in cell wall elongation, and thus root growth, which fundamentally enhances plant growth under salt tolerance.

In general, the root cell wall plays a crucial role in regulating root growth, and

that differences in the properties of the root cell wall among different cultivars can affect their tolerance to saline stress. Specifically, characteristics of root cell wall such as higher amounts of uronic acids and pectin, higher amounts of cellulose, and demethylation and increased side chains of pectin were of importance for root growth under salinity stress.

This study explored the relationship between physical and chemical properties of the root cell wall and the effects of pectin on the salt tolerance of spinach. The focus was on understanding how cell wall pectin characteristics can affect plant growth under salinity stress. The findings provide insight into plant salt tolerant mechanism and have potential applications for improving plant growth under salinity stress.

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Summary

Soil salinization is one of the serious environmental factors limiting agricultural productivity. Improving crop salt tolerance on the basis of tolerance mechanisms will ultimately contribute to the crop production in the world. The growth response of halophytes and glycophytes to salinity can differ significantly. The study of the physiological responses of glycophytes and halophytes to salinity is useful for understanding the mechanisms underlying plant salinity tolerance. Recent studies on the mechanism of salt tolerance in plants have focused on the symplast pathway, while studies on the apoplast pathway are relatively limited. Root cell wall directly interacts with the salts present in the soil and plant. But the functions of root cell wall in plant salt tolerance are still unclear.

In this study we investigated cell wall composition, extensibility, and viscosity in the root elongation zone of young seedlings of halophyte (*Suaeda salsa*) and glycophyte (*Spinach oleracea* cv. 'Akinokagayaku'), which both belong to the Amaranthaceae family. Furthermore, we investigated the salinity tolerance of three spinach cultivars (Helan 3, Prius β and R7) with a focus on pectin content, such as: pectin polysaccharides, the degree of pectin methy-lesterification (PMD) and pectin-related wall parameters in the cell walls. The objectives of this study were elucidating the interactions of cell wall composition, extensibility and root growth under salinity stress and pectin characteristics of root cell wall that contribute to root growth under salinity.

1. Chemical composition of root cell wall in relation with cell wall extensibility and root growth under salinity stress

Two days after germination, young seedlings of *Suaeda salsa* and *Spinach oleracea* were treated with a series concentration of NaCl (0, 100, 200 and 300 mM). After the seedlings were subjected to the salinity treatments for 8 days, root samples were collected and relative parameters were determined.

Root growth was inhibited by increased salinity in both species. The pronounced inhibition in the root growth in *S. oleracea* grown under salt stress showed relatively greater sensitivity to salinity when compared with *S. salsa*. The cell wall contents were much less in *S. oleracea* than *S. salsa*. With the increase in NaCl concentration,

pectin contents were significantly decreased in *S. oleracea*. There was a significantly positive correlation between pectin contents and root growth in this species. In *S. salsa*, there was no decrease in the pectin content with salinity treatments. There was no difference in the contents of hemicellulose I or II in *S. oleracea* among all treatments. In *S. salsa*, there was a significant increase in the contents of hemicellulose I and II under 300 mM NaCl condition. Cellulose contents did not change after salinity treatments in both species. Salt treatments decreased the uronic acid contents in pectin fraction in *S. oleracea*. There was a significantly positive correlation between uronic acid contents in pectin fraction and root growth. In *S. salsa*, the uronic acid contents in pectin fraction were maintained at 100 mM NaCl but significantly decreased when NaCl concentrations elevated above 200 mM. In general, the tendencies of uronic acid in each cell wall fractions were similar to those fractions in response to the series of salinity treatments in either of the species. These results suggest that cell wall pectin plays important roles in root growth in both species under salinity, and that the salt tolerance of glycophyte *S. oleracea* is affected by the pectin. Cellulose limits root elongation under saline conditions in both species, but in halophytes, a high cell wall content and the proportion of cellulose in cell walls may be a salt tolerance mechanism that protects the stability of cell structure under salt stress.

Salinity treatments decreased the cell wall extensibility in *S. oleracea*. There was a significantly positive correlation between the extensibility and root growth in this species. NaCl, up to 200 mM, increased the cell wall extensibility in *S. salsa*. Under 200 mM NaCl concentrations, cell wall extensibility was significantly higher in *S. salsa* as compared with *S. oleracea*. The similar tendency could be observed in the viscosity of root cell wall of the two species. The negative correlation observed in this study between E_0 and root growth in *S. salsa* and *S. oleracea* indicates that cell extensibility in the root elongation zone is an important limiting factor of root growth in both halophyte and glycophyte species under saline conditions.

In *S. salsa*, the plastic extension showed negative values, except in the 200 mM treatment. The negative value of plastic extension means that the root shrunk even shorter than the original length after extension. In contrast, this phenomenon was not found in *S. oleracea.* To date, there are no reports of root shrinkage exceeding the original measured root length, indicating a negative plastic deformation in halophyte

roots. This phenomenon might be due to the high cellulose content of the cell walls in this species. Cellulose chains can expand in a direction that is perpendicular to the cellulose chains after a tensile force is applied. This response of the cellulose chains to tensile force may have caused the vertical shrinkage observed in the *S. salsa* roots. A high amount of cellulose may provide tensile strength and crosslink sites that increase the cell wall stiffness and ensure the cell structure of *S. salsa* under salinity stress.

2. Pectin characteristics in relation with cell wall extensibility and root growth in spinach under salinity stress

Two days after germination, young seedlings of spinach cultivars, Helan 3, Prius β and R7 were treated with a series concentration of 0 and 200 Mm NaCl. After the seedlings were subjected to the salinity treatments for 6 days, root samples were collected, and relative parameters were determined.

Cultivar R7 showed higher root growth under salinity stress compared with Helan 3 and Prius β. This indicates that the R7 had higher salt tolerance than the other two cultivars. The pectin content was significantly increased across the cultivars under salinity stress, whereas the molar proportion of uronic acid in pectin was significantly decreased in Helan 3 and R7 cultivars. There was a positive correlation between the molar proportion of uronic acid in pectin and the root length of the two cultivars. This result implies that pectic uronic acid was consistent with root growth in the two cultivars. A similar trend was observed in *S. salsa* and *S. oleracea* 'Akinokagayaku'. These results indicate that pectic uronic acid plays important roles in root growth in both species under salinity.

Salinity significantly reduced cell wall extensibility in all cultivars, and increased cell wall viscosity in Helan 3 and R7 relative to Prius β. Pectin content, the molar proportion of uronic acid in pectin and HG:RG-I ratio were significantly correlated with E_0 under salinity stress in Helan 3 and R7 cultivars. These correlations indicate that cell wall extensibility was affected by pectin in Helan 3 and R7, which may have affected cell expansion under the stress condition. The molar proportion of uronic acid in pectin and the HG:RG-I ratio were significantly correlated with cell wall viscosity across the cultivars; this is an indication that uronic acid in pectin and the RG-I backbone may have regulated cell wall viscosity during salinity stress in spinach.

Compared with Helan 3 and Prius β, the high salt tolerance of the R7 was significantly correlated with the pectin characteristics. The length and degree of pectin methy-lesterification of neutral side chains were significantly decreased in the R7 cultivar, with no significant changes in the other two cultivars. The demethylation and increased side chains of pectin under salinity stress may lead to changes in cell wall elongation, and thus root growth, which fundamentally enhances plant growth under salt tolerance.

This study has shown that the root cell wall plays a crucial role in regulating root growth, and that differences in the properties of the root cell wall among different cultivars can affect their tolerance to saline stress. Specifically, characteristics of root cell wall such as higher amounts of uronic acids and pectin, higher amounts of cellulose, and demethylation and increased side chains of pectin were of importance for root growth under salinity stress.

論文要旨

土壌塩類化は、農業生産性を制限する深刻な環境要因の 1 つである。耐性メカニズムに基づいて作物の耐塩性を改善することは、最終的には世界の作物生産に貢献する。塩生植物と非塩生植物の塩分に対する成長反応は、大きく異なる可能性がある。塩分に対する塩生と非塩生植物の生理学的反応の研究は、植物の塩分耐性の根底にあるメカニズムを理解するのに役立つ。植物の耐塩性のメカニズムに関する最近の研究ではシンプラスト経路に焦点を当てられてきたが、アポプラスト経路に関する研究は比較的限られている。根の細胞壁は、土壌や植物に存在する塩と直接相互作用する。しかし、植物の耐塩性における根の細胞壁の機能はまだ不明である。

本研究では、両方ともヒユ科に属する塩生植物(*Suaeda salsa*)と非塩生植物(*Spinach oleracea* cv. 'Akinokagayaku')の幼苗の根の伸長部における細胞壁の組成、伸長性、および粘性を調査した。さらに、ペクチン多糖類、ペクチンメチルエステル化(PMD)の程度、およびペクチン関連の細胞壁特性を調査した。本研究の目的は、塩分ストレス下での細胞壁組成、伸長性、根の成長の相互作用、および塩分下での根の成長に寄与する根の細胞壁のペクチン特性を解明することである。

1.塩分ストレス下での根の細胞壁の化学組成と細胞壁の伸長性および根の成長の関係

発芽の 2 日後に *S. salsa* と *S. oleracea* の実生を NaCl (0、100、200、および 300 mM)で処理した。実生を 8 日間塩処理した後、根のサンプルを採取し、相対パラメータを測定した。両方の種で根の成長は塩分濃度の増加によって阻害された。*S. salsa* と比較して、塩ストレス下で生育させた *S. oleracea* では根の成長が顕著に阻害され、塩分に対する感受性が比較的高いことが示された。*S. oleracea* では、*S. salsa* よりも細胞壁の含有量が顕著に少なかった。 *S. oleracea* では、NaCl 濃度の増加に伴い、ペクチン含有量が顕著に減少した。この品種のペクチン含有量と根の成長との間には有意な正の相関が認められた。*S. salsa* では、塩処理によるペクチン含有量は減少しなかった。*S. oleracea* のヘミセルロース I または II の含有量は、すべての処理で差がなかった。*S. salsa* では、300 mM NaCl 条件下でヘミセルロース I および II の含有量が顕著に増加した。セルロース含有量は、両方の種で塩処理後も変化しなかった。塩処理は *S. oleracea* のペクチン成分のウロン酸含有量を減少させた。ペクチン分画中のウロン酸含有量と根の成長との間には有意な正の相関が認められた。*S. salsa* では、ペクチン画分のウロン酸含有量は 100 mM NaCl で維持されたが、NaCl 濃度が 200 mM 以上では顕著に減少した。一般的に、各細胞壁画分のウロン酸の傾向は、いずれかの種においても一連の塩処理に対応した画分と同様であった。これらの結果は、細胞壁ペクチンが塩分条件下における両種の根の成

長に重要な役割を果たしており、非塩生植物 *S. oleracea* の耐塩性はペクチンによって影響を受けることが示唆された。セルロースは両種とも塩処理条件下での根の伸長を制限するが、塩生植物では、細胞壁の含有量が多く、細胞壁中のセルロースの割合が高いことが、塩ストレス下で細胞構造の安定性を保つ耐塩性メカニズムである可能性がある。

塩処理は、*S. oleracea* の細胞壁の伸展性を低下させた。この種の伸長性と根の成長の間には有意な正の相関が認められた。一方、最大 200 mM の NaCl 濃度では、*S. salsa* の細胞壁の伸長性を高めた。200 mM の NaCl 濃度では、*S. oleracea* と比較して、*S. salsa* の細胞壁の伸長性が有意に高かった。両種の根細胞壁の粘性にも同様の傾向が見られた。本研究で観察された *S. salsa* と *S. oleracea* の E_0 と根の成長との負の相関は、塩処理条件下における塩生植物と非塩生植物の両方の種で、根の伸長部での細胞の伸長性が根の成長にとって重要な制限要因であることを示している。

S. salsa では、200 mM の NaCl 処理を除いて、可塑性伸長は負の値を示した。可塑性伸長の負の値は、根が伸長後に元の長さよりもさらに短く収縮したことを意味する。対照的に、この現象は *S. oleracea* では見られなかった。これまでに、測定された元の根の長さを超える根の縮小が報告されていない。これは、塩生植物の根の負の塑性変形を示している。この現象は、この種の細胞壁のセルロース含有量が高いためである可能性がある。セルロース鎖は、引張力が加えられた後、セルロース鎖に垂直な方向に拡張できる。このような引張力に対するセルロース鎖の応答は、*S. salsa* の根で観察された垂直方向の収縮を引き起こした可能性がある。多量のセルロースは、塩分ストレス下における *S. salsa* の細胞壁の剛性を高め、細胞構造を維持するための引張強度と架橋部位に寄与する可能性がある。

2.塩分ストレス下におけるホウレンソウの細胞壁伸長性と根の成長に関連するペクチンの特性

発芽の 2 日後、ホウレンソウ品種 Helan 3、Prius β、および R7 の幼実生を、0 および 200 Mm NaCl の一連の濃度で処理した。実生を 6 日間塩処理した後、根のサンプルを採取し、相対パラメータを測定した。

栽培品種 R7 は、Helan 3 および Prius β と比較して、塩分ストレス下でより高い根の成長を示した。これは、R7 品種が他の 2 品種よりも耐塩性が高いことを示している。ペクチン含有量は、塩分ストレス条件下で品種間で顕著に増加したが、ペクチン中のウロン酸のモル比率は Helan 3 および R7 品種で大幅に減少した。ペクチン中のウロン酸のモル比率と 2 つの品種の根の長さの間には正の相関が認められた。この結果は、ペクチンウロン酸が 2 つの品種の根の成長と一致することを示している。*S. salsa* と *S. oleracea* ‘Akinokagayaku’でも同様の傾向が観察された。これらの結果は、ペクチンウロン酸が塩分下で両種の根の成長に重要な役割を果たしていることを示している。

塩分濃度は、すべての品種で細胞壁の伸長性を大幅に低下させ、Prius β と比較して Helan 3 と R7 で細胞壁の粘度を増加させた。ペクチン含有量、ペクチン中のウロン酸のモル比率、および HG:RG-I 比は、Helan 3 および R7 品種の塩分ストレス下で伸張性との有意な相関が認められた。これらの相関関係は、Helan 3 および R7 品種のペクチンが細胞壁の伸長性に影響を与えたことを示しており、ストレス条件下での細胞の伸長に影響を与えた可能性がある。ペクチン中のウロン酸のモル比率と HG:RG-I 比は、品種全体で細胞壁の粘度と有意な相関が認められた。これは、ペクチンのウロン酸と RG-I 骨格が、ホウレンソウの塩分ストレス時に細胞壁の粘性を制御した可能性を示唆するものである。Helan 3 および Prius β 品種と比較して、R7 品種の高い耐塩性は、ペクチンの特性と有意な相関が認められた。中性側鎖のペクチンメチルエステル化の長さとその程度は、R7 品種で有意に減少したが、他の 2 品種では有意な変化はなかった。塩分ストレス下におけるペクチンの脱メチル化と側鎖の増加は、細胞壁の伸長の変化、ひいては根の伸長に変化をもたらし、塩耐性下での植物の成長を根本的に促進させると考えられる。

本研究により、根の細胞壁が根の成長を制御する上で重要な役割を果たすこと、また、異なる品種間での根の細胞壁の特性の違いが塩ストレスに対する耐性に影響を及ぼす可能性があることが明らかになった。具体的には、ウロン酸とペクチンの含有量が多いこと、セルロースの含有量が多いこと、ペクチンの脱メチル化と側鎖の増加などの根の細胞壁の特性が、塩分ストレス下での根の成長に重要であることがわかった。

Publication list

Paper 1:

Title:

Cell Wall Components and Extensibility Regulate Root Growth in *Suaeda salsa* and *Spinacia oleracea* under Salinity

Authorship:

Jia Liu, Yang Shao, Xiaohui Feng, Victoria Otie, Asana Matsuura, Muhammad Irshad, Yuanrun Zheng, Ping An

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