WILLIAM ROLAND LEATHERWOOD. Influence of salt stress on germination, root elongation and carbohydrate content of five salt tolerant and sensitive taxa. (Under the Direction of John David Williamson.)

Because salt stress can reduce photosynthesis and yields as well as reduce crop value by damaging appearance, field, container, and greenhouse producers must manage salinity carefully to assure a profitable crop. Understanding plant responses to salt stress is key in managing this problem. A common protective response to salt stress is osmoregulation and/or osmoprotection via the accumulation of carbohydrates or compatible solutes such as the sugar alcohol mannitol. Various protective roles for these compatible solutes have been well documented in plants, cell cultures and maturing seed. Less information is available on possible impacts of these compounds on protection against salt stress during the germination process itself. To specifically assess potential roles for sugars and sugar alcohols on germination, growth and carbohydrate metabolism, we selected seed from known mannitol and non-mannitol accumulating salt tolerant taxa and seed from known salt sensitive non-mannitol accumulating taxa, and germinated them in the presence of increasing concentrations of sodium chloride.

Seed of both salt tolerant and sensitive taxa showed varying degrees of decreased radicle elongation as salt stress increased. Surprisingly, seed from taxa known to accumulate mannitol did not germinate better in saline environments than did seed from non-mannitol producing taxa. In fact, seed from non-mannitol taxa described as salt sensitive had somewhat higher germination and better growth under salt stress than seed of salt tolerant mannitol-containing plants. Analysis of soluble carbohydrates in seeds and seedlings

incubated in the presence of increasing salt showed no overall correlation between mannitol content and germination or growth. However, accumulations of low molecular weight carbohydrates, especially sucrose, were observed at high levels of salt stress. Interestingly, this increase most often occurred after concentrations of salt were reached that totally inhibited germination, suggesting a potential role for carbohydrates in conditioning of seed during salt stress.

Influence of salt stress on germination, root elongation and carbohydrate content of five salt tolerant and sensitive taxa.

by

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“For those who teach

and seek”

Biography

William Roland Leatherwood was born November 7, 1969 to Richard Keith and Heidi Impertro Leatherwood. Roland's university education began in August 1988 at the University of North Carolina Asheville where he received a Bachelor of Arts degree in German and Bachelor of Science degrees in Economics and Management. After working in public mental health for several years, Roland elected to attend North Carolina State University to pursue an advanced degree in Horticulture. While taking preparatory post-baccalaureate classes, Roland worked closely with Dr. Eric Davies isolating polysomes from gravistimulated corn roots. In August 2002, Roland began his graduate education as a Masters student in the Department of Horticultural Science at North Carolina State University under the direction of Dr. John Williamson.

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Literature Review

Influence of salt stress on germination, root elongation and carbohydrate content of five salt tolerant and sensitive taxa.

The Problem of Salinity

Saline soils are a common limiting factor for agricultural productivity in semiarid and arid areas of the world. Worldwide it is estimated that 33% of irrigated lands are affected by salinization and certainly more land passes out of irrigation due to salinization as new land becomes available. Soil salinization is a widespread problem geographically with an impact that spans recorded history. Not only is it hypothesized that many ancient agrarian societies collapsed due to soil salinization (Rhodes, 1990), but large areas of the Indian subcontinent have recently been made useless for crops due to this problem (see Marschner, 1998).

Human activity has increased the hectares of saline soils. The use of slightly saline irrigation water can lead to a several fold increase in the salinity of the soil solution. Repeated irrigation through the season in combination with transpiration and evaporation leave behind salts, thus increasing soil salinity. As a result, agricultural areas from the Mediterranean to southwestern North America face continuing problems with soil salinization.

Movement of underground saline water in combination with insufficient rainwater for leaching can create saline soils (see Marschner, 1998). The problem of seawater incursion

into shallow groundwater is largely due to human activity and the impact on crop production is under continuing research (Vengosh and Rosenthal, 1994). In eastern North Carolina, saltwater incursion into freshwater aquifers has recently become a problem as agricultural, domestic and commercial demand for freshwater climbs (Volosin and Spruill, 2001).

Finally, localized areas of ion toxicity can develop due to abandoned mine spoils. For example the decomposition of sulfidic shale and copper mine waste in the western mountains of North Carolina has created areas containing toxic amounts of aluminum (Hammarstrom et al., 2003).

Salinity and Horticultural Crops

While perhaps less extensive in terms of hectares impacted, soil salinization is also of concern to horticulturists. Citrus, for example, is commonly grown in semi-arid regions and is salt sensitive. Salinity can have a negative impact on the nutrient relationship of the plant causing fruit damage in several vegetable crops (Schnitzler and Gruda, 2002). Development of salt tolerant vegetables such as tomato *Solanum lycopersicum* (L.) *cerasiforme* (Dunal) Spooner, J. Anderson & R.K. Jansen, by traditional breeding techniques has been slow due to the complexity of the desired trait (Foolad, 2004). Strawberry *Fragaria* L. shows a reduction in productivity and leaf area proportional to increasing levels of salt stress (Giuffrida et al., 2001).

In nursery and greenhouse production incorrect watering combined with the solubilization of fertilizers can lead to increased ionic stress causing bud and leaf drop (ter Hell and Hendriks, 1995). Minor changes in electrical conductivity and salinity in a

hydroponic system can delay a crop or cause poor yields. For example, *Dianthus caryophyllus* L. and *Gerbera jamesonii* L. show reduced size and number of flowers in very slightly saline environments.

Physiological and Cellular Impacts of Salt Stress

Specific effects of increased soil salinity on plants can be quite diverse. Not only do specific ions have particular impacts on cellular processes but salt stress imposes many of the same limitations on biological processes as a shortage of water (Bohnert et al., 1995; Bray, 1997). For simplicity, ionic and osmotic effects will be considered separately.

Effects of Ion Toxicity

Sodium and other ions can disrupt proteins by passing through the sphere of hydration and interfering with hydrogen bonds and Van der Waals interactions. The result is denaturation and loss of function. Depending on the number of such bonds stabilizing a protein's structure, different proteins will denature and become insoluble at different salt concentrations. This is commonly used in the laboratory as a first step in many protein purification protocols.

Sodium binds to photosystem two (PSII) and irreversibly blocks the oxygen evolving machinery thus inhibiting electron transfer from water (Allakhverdiev et al., 2000). The resulting generation of reactive oxygen species (ROS) from PSII in turn rapidly disrupts associated protein and membrane structures. Evidence of such damage appears as necrotic lesions on leaves.

In *Vicia fava* L. stomatal guard cells, sodium blocks the outward rectifying K+ channel current (Thiel and Blatt, 1991) thus preventing the ABA and calcium dependent signalling mechanism from closing the stomatal pore. Eventually, the inability of the cell to cope with impaired K+ channel operation slows growth, or at higher levels, leads to plant death.

Aluminum toxicity is of growing concern throughout the southeast. Not only cropland, but also forests are affected as the increasing frequency of acid rain changes soil chemistry and leads to the solubilizing of aluminum ions (Huntington et al., 2000). Aluminum has long been known to inhibit root growth in many species. Research into aluminum toxicity using squash, *Cucurbita moschata* (Duchesne ex Lam.) Duchesne ex Poir., found that ascorbate concentrations in the root meristems decreased when exposed to toxic amounts of aluminum. When amounts of ascorbate in the rhizosphere of treated plants were artificially increased by the addition of boron, root growth recovered (Lukaszewski and Blevins, 1996). Other researchers have verified that many plants actually exude ascorbate and other organic acids into the rhizosphere to chelate aluminum ions before they enter the symplast. (Delhaize et al., 2004; Kochian et al., 2002). Investigators researching the mechanism of aluminum toxicity found that aluminum ions disrupt cell signalling involved in many stress responses (Kopka et al., 1998) and that aluminum interferes with H^+-ATPase activity by permanently disrupting the polarization of the vacuolar plasma membrane (Ahn et al., 2001).

Frequently, necessary micronutrients are toxic at higher concentrations. Copper, manganese, lead and cadmium, among others, create a highly consistent pattern of damage in

plant cells. Copper ions interfere with membrane integrity causing leakage, and stimulate the generation of free radicals. Manganese, lead and cadmium change protein conformation by complexing with sulphydryl groups. These ions can also displace other elements in macromolecules and enzymes changing their conformation and impairing function (Epstein and Bloom, 2005).

Osmotic Effects of Salt Stress

As indicated earlier, salt stress, besides specific ion effects, can induce osmotic stress. Under mild water stress the stomata of temperate C3 plants close, reducing carbon dioxide uptake. The resulting shortage changes the ratio of O_2 to CO_2 in the chloroplast and increases photorespiration. Phloem loading of carbohydrates slows because of slowed photosynthate production.

Conversely, increases in specific ion concentration due to water loss can interfere with various plant processes. For example, increased levels of magnesium (Mg^{2+}), a key electron transfer mediating component of chlorophyll, interferes with photosynthesis, lowering the rate of photosynthetic production in water stressed sunflower (Rao et al., 1987).

Photosynthesis does not occur within germinating seed, so a source of free electrons generated under saline conditions is absent. Germinating seed is capable of tolerating near complete desiccation up to the point of radicle elongation. In the early hours of imbibition membranes are leaky and various low molecular weight substances, ions, sugars and amino acids leach out into the surrounding medium or into seed tissues.

After the first hours of imbibition membrane integrity is restored, organelles repaired and protein synthesis begins. In grain crops such as barley *Hordeum vulgare* L., gibberellic acid (GA) from the embryo induces α-amylase synthesis, which is responsible for activating hydrolysis of starch. After repair of membranes and reactivation of organelles, mobilization of carbohydrates occurs as sugars from the endosperm are transported to the embryo (e.g. see Bewley, 1997). Proteolysis of extant proteins in the endosperm by aminopeptidases or endopeptidases allows the translocation of resulting amino acids to growing points for new protein synthesis or nitrogen scavenging. Finally, lipids hydrolyzed by lipases yield fatty acids and glycerol. Carbon from these compounds moves from the oleosome to the glyoxysome then to the mitochondrion and finally to the cytosol for gluconeogenesis.

The process of germination is intimately dependent on protein function, yet is surprisingly robust in terms of salt stress and desiccation tolerance. Many seeds can complete the early processes of germination and be re-desiccated without damage. Known as seed priming, this process is used to produce seed that germinates faster and more uniformly after treatment (Parera and Cantliffe, 1994).

Plant Responses to Salt and Osmotic Stress

Plants deal with salt and osmotic stress by adaptation and/or acclimation. Adaptation is the result of permanent genetic changes, applies to the whole species, is usually part of the normal life process of the plant and is "constitutive" (i.e. the photosynthetic stems of cacti are expressed regardless of stress conditions). In contrast acclimation is a response by individual plants, is not part of the "normal" life cycle and is not constitutive (i.e. is

reversible after the inducing signal is removed). The sharp increase in proline concentration in cold stressed spinach *Spinacia oleracea* L. is an example.

Whether adaptation or acclimation, both mechanisms can be further classified as either avoidance or tolerance responses. For example, the adaptive mechanism of seed dormancy allows the seed to avoid temperature or drought stress. On the other hand, the induction of a repair mechanism in response to photooxidative stress is a tolerance response.

Three primary tasks plants have in dealing with salt and osmotic stresses are taking up available water against an osmotic gradient, maintaining water balance and maintaining appropriate levels of essential ions. Specific cellular manifestations of these responses are highly conserved across phyla, with similar mechanisms being found in algae, fungi, bacteria, vascular and nonvascular plants (Csonka, 1989; Del Moral et al., 1994; Potts, 1994).

Whole Plant Responses

Many physiological responses in vascular plants are designed to maintain or increase water uptake, reduce water loss and mitigate osmotic and ionic stress. Typically the most rapid response to osmotic stress, induced either by drought or increased salinity, is to minimize water loss by closing stomata. Unfortunately, under prolonged water stress this blocks gas exchange and thus both the uptake of CO_2 and the release of O_2. This results in increased O_2 to CO_2 ratios which favor the photorespiratory reactions of Rubisco over CO_2 fixation (see Taiz and Zeiger, 1998). Plants have adapted to these conditions by evolving alternate photosynthetic strategies such as C4 and CAM photosynthesis.

Plants may also minimize water loss by physically reducing the leaf surface area. Decrease in leaf area can include growing smaller leaves, but these are less able to cool the plant through transpiration. Complete abscission of leaves and dormancy are also responses to long-term osmotic stress. While these responses reduce transpiration they significantly reduce photosynthesis as well. Under extreme conditions adapted plants may evolve photosynthetic stems, which negate the need for leaves entirely. Some adapted plants increase wax deposition on their leaves or have hirsute leaves to minimize transpiration.

Plants respond to osmotic stress not only by preventing loss but also by increasing water uptake. There is some evidence that within the xylem intervessel pit vestures, porous flexible membranes covering intervessel pits alongside xylem cells, are able to create a negative pressure which aids in maintaining the upward flow of the xylem sap (Choat et al., 2004). Longer-term osmotic stress promotes root growth to reach deep soil moisture. Plants with deeper roots use hydraulic lift to bring deep soil moisture to the upper levels. Deep soil water is released from the shallow roots to dissolve nutrients and is then actively reacquired (Epstein and Bloom, 2005).

The integration of cellular responses with whole plant mechanisms is critical. For example, water deficit perceived by the root endodermal cells stimulates ABA synthesis and transport. Changes in ABA content of the shoots, in turn, cause shoot-specific responses in epidermal cells and stomata (Bray, 1997). Mechanisms such as ABA signalling also regulate other cellular responses.

Cellular Mechanisms for Dealing with Salt Stress

Specific cellular responses to salt and osmotic stresses are numerous and often quite complex. A comprehensive review of these responses is beyond the scope of this chapter, so only a few representative mechanisms will be discussed.

Aquaporins are some of the best known stress response proteins. These are selective membrane channels that facilitate water movement (Schaeffner, 1998). Changes in the rate of movement of water through aquaporins is regulated in response to a variety of signals by specific changes in protein phosphorylation (Maurel et al., 1997). For example, the *Phaseolus vulgaris* L. α-TIP, a tonoplast intrinsic protein in germinating seed, is up regulated by a Ca^{2+} dependent protein kinase (CDPK, Maurel et al., 1995). Conversely, ionic stress decreases aquaporin permeability in corn *Zea mays* (L.) ssp. *mays* roots, presumably to facilitate the exclusion of harmful ion species (Baiges et al., 2002).

Another class of proteins involved with cellular responses to salt and osmotic stresses are the late embryogenesis abundant (LEA) proteins. These are expressed during the final stages of embryo development and are thought to help in developing desiccation tolerance in seed. The putative range of functions is large and includes binding and replacement of water, membrane stabilization, maintenance of membrane and protein structure, and ion sequestration (Figueras et al., 2004). For example, Rab17 from corn accumulates in the late stages of embryo formation and in vegetative tissues enduring stress conditions. Transgenic *Rab17* over expressing Arabidopsis plants subjected to osmotic stress accumulate proline and sugars at a higher rate than controls and recover more quickly from both salt and osmotic stress (Figueras et al., 2004). On the other hand, Rab16 in rice was found to be responsive to

water stress but not salt stress, suggesting that the protein is involved in drought response of germinating seedlings (Rao et al., 1993).

Another group of proteins involved in ionic stress response are specific ion transporters. Several active transporters for sodium uptake and extrusion across the plasmalemma have been identified. Other sodium transporters in the tonoplast allow sodium sequestration from the cytoplasm (Blumwald et al., 2000). These mechanisms have been found in numerous organisms including yeast, tobacco, blue-green algae, red beets, barley, tomato and corn (Blumwald et al., 2000). To balance the solute potential differences between vacuole and cytoplasm, osmolyte concentrations in the cytoplasm are increased. Another method of avoiding ion toxicity is to prevent the offending compound from entering the plant at all or to secrete concentrated salts. Squash is known to cope with toxic levels of aluminum by exuding ascorbate which chelates the ion in the soil, thus avoiding the problem of toxicity (Lukaszewski and Blevins, 1996). Some grasses and members of the *Aizoaceae* exude excess salts via salt glands in the leaves (Marcum 1999, Marcum et al., 1998).

Compatible Solutes

Among the most studied stress metabolites are compatible solutes. They are low molecular weight compounds that do not interfere with cellular processes even at high concentrations. Compatible solutes include such diverse compounds as polyhydric alcohols, amino acids, tertiary sulfonium compounds, and quaternary ammonium compounds. These compounds can act as osmolytes, shifting water potential gradients, or as osmoprotectants, maintaining a sphere of hydration around proteins.

Plant cells modulate concentrations of compatible solutes via active transport of water or by synthesis, to counter osmotic changes. Compatible solutes can be rapidly derived from complex macromolecules while under stress, and then removed by rapid repolymerization when the stress is gone. A balance of catabolism and anabolism maintains appropriate concentrations (Bray et al., 2000).

Compatible solutes are also hypothesized to protect proteins by maintaining a sphere of hydration. Many compatible solutes are zwitterions at physiological pH and thus have slightly positive and negative ends. These ends orient to maintain a sheath of water around proteins, or may themselves directly stabilize proteins in ways similar to water. Compatible solutes are similarly hypothesized to stabilize membranes. For example, in osmotically stressed germinating seed, free sugars accumulate to high levels (Thind, 1991). This observation is in accord with the fact that similar accumulation of soluble carbohydrates stabilizes membranes in maturing seed (Obendorf ,1997).

Still other compatible solutes are amino acids and their derivatives. The amino acid proline accumulates in plants subjected to salt or osmotic stress. Its accumulation is dependent on the expression of both Δ^1-pyrroline-5-carboxylate synthase (P5CS) and proline dehydrogenase (PDH) (Coruzzi and Last, 2000). Proline transport is also up regulated under salt stress (Rentsch et al., 1996) and screening for such a response may be a way to find stress tolerant breeding stock in citrus (Mademba-Sy et al., 2003) and barley (Pakniyat et al., 2003).

Glycine betaine, an amino acid analog, is synthesized from betaine aldehyde by betaine aldehyde dehydrogenase (BADH-1). It accumulates to high levels in the leaves of

water stressed bean plants. Interestingly, when exogenously applied, water stressed plants maintain turgor, photosynthetic efficiency and recover from water stress more rapidly than control plants. (Xing and Rajashekar, 1999). Further investigations into glycine betaine's stress response role found that *BADH-1* transformed tomato plants were able to maintain appropriate root water potential under salt stress conditions compared to control plants (Moghaieb et al., 2000). Other investigators found glycine betaine's ability to increase stress tolerance is at least partially due to stabilization and accelerated repair of photosynthetic proteins. Measurements of photosynthesis in transformed plants placed under cold, light and salt stress, show unchanged or only moderately reduced photosynthetic rates compared to wild type (Sakamoto and Murata, 2002).

Another compound reported to improve chloroplast stress tolerance is β-dimethylsulfoniopropionate (DMSP). DMSP is synthesized in the chloroplast from imported cytosolic S-methylmethionine (SMM). The methionine to DMSP biosynthetic pathway has evolved independently three times and this diverse biochemistry offers appealing targets for transgenic engineering of stress tolerant plants. It is also worthy to note, that unlike glycine betaine and proline, DMSP contains no nitrogen (McNeil et al., 1999). DMSP's role in stress tolerance is quite similar to glycine betaine's. When the levels of DMSP and SMM in the chloroplast and cytosol were compared in salt treated and control *Wollastonia biflora* (L.) DC. plants, a significant increase of DMSP in the chloroplast compared to the cytosol was observed in the salt stressed plants. The difference is enough to protect the plastid from stress injury and maintain osmoregulation. Chloroplastic SMM levels accounted for 40% of the overall cellular amounts in unstressed plants, but increased to 80% under salt stress

(Trossat et al., 1998). The result is a clear indicator of DMSP's role in protecting chloroplasts during osmotic stress.

Carbohydrates

Simple Sugars

Carbohydrates are products of photosynthesis and readily available osmolytes. Simple sugars such as sucrose, fructose, and glucose all act as osmolytes and / or compatible solutes and can be quickly synthesized from polymeric forms in response to stress. During germination, raffinose and starches are converted to glucose or other simple sugars. The enzyme α-amylase releases monomeric sugars from starch. A drought tolerant cultivar of chickpea, *Cicer arietinum* L., shows higher α-amylase activity in cotyledons of germinating seed than a drought sensitive cultivar (Gupta et al., 1993). The greater concentration of monomeric sugars in the drought tolerant variety acts to adjust osmotic potential.

Multiple carbohydrate types can also simultaneously impact stress tolerance as seen in the metabolism of the resurrection plant, *Craterostigma plantagineum* Hochst (Bartels, 1990). Adult plants undergo a reversible partitioning of carbohydrate during growth and desiccation. Well watered plants accumulate 2-octulose, an eight carbon carbohydrate, and during desiccation convert it to sucrose for stabilizing membranes (Norwood et al., 2000). A related mechanism is observed in maturing seed of *Pisum sativum* L. where sucrose is converted to raffinose oligosaccharides during desiccation (Peterbauer et al., 2001) which are also thought to stabilize membranes.

Raffinose Family Oligosaccharides

Raffinose family oligosaccharides (RFOs) include a number of complex carbohydrates such as stachyose and galactinol. RFOs have an established role in desiccation tolerance of seed and are the second most common storage carbohydrate in seed. They are also rapidly converted during germination to simple sugars.

It has been suggested that RFOs protect seeds against damage during desiccation and aging, which increases storability and longevity. One hypothesis is that RFOs interact with cell membranes in the area of the phospholipid head groups, thus increasing their stability during desiccation. Studies with corn, *Zea mays* L., show that RFOs and monomeric sugars together are able to form highly vitreous states that physically stabilize cellular membrane components. There is also a positive correlation between the formation of this glassy state and seed storage stability (Obendorf ,1997).

During osmotic or salt stress, Arabidopsis accumulates high levels of raffinose and galactinol compared to controls. Two salt and drought induced galactinol synthase genes (*GolS)* when over expressed in transgenic Arabidopsis resulted in plants that were able to tolerate higher levels of salt and water stress than wild type (Taji et al., 2002). In addition, plants over-expressing the drought response factors DREBA1A/CBF3 have been found more tolerant of dehydration (Kasuga et al., 1999) and contained increased concentrations of sucrose, glucose, fructose, raffinose and other sugars (Gilmour et al., 2000). These results confirm a direct role for raffinose and other sugars as osmoprotectants and osmolytes.

Polyols

Polyols are reduced forms of corresponding sugars (i.e. sugar alcohols) and act as osmolytes, osmoprotectants and antioxidants. The correlation between salt and osmotic stress tolerance and polyol accumulation is well established (Loewus and Dickinson, 1982).

Pinitol, 1D-3*O*-methyl-chiro-inositol, is known to confer drought tolerance in *Glycine max* L., soybean, where pinitol to sucrose ratios increase with increasing stress (Guo and Oosterhuis, 1997). Breeders searching for high pinitol accumulators have repeatedly confirmed the correlation between pinitol content and water stress tolerance (Streeter et al., 2001). In *Cercis canadensis,* levels of pinitol, myo-inositol and the methylated inositol D-ononitol increase under heat and water stress (Griffin et al., 2004).

In *Mesembryanthemum crystallinum* L., ononitol is a key stress related polyol. L-myo-inositol 1-phosphate synthase (INPS) catalyzes the first step of ononitol synthesis from glucose-6-phosphate. Myo-inositol 6-*O*-methyltransferase (IMT) converts myo-inositol to ononitol. During normal growth, INPS levels are steady in *M. crystallinum* and expressed in all types of cells while IMT is repressed. Salt stress causes up regulation of INPS activity in the leaves and down regulation in the roots while IMT is induced in all cell types. Phloem transport of the INPS product, myo-inositol, facilitates the accumulation of ononitol and related compatible solutes, allowing for the protection of photosynthesis and sequestration of sodium (Nelson et al., 1998). These results were confirmed in transgenic studies wherein *Imt* over-expressing tobacco transformants showed higher levels of CO_2 fixation and faster recovery than wild type plants when placed under osmotic stress. Ononitol levels increased steadily over the entire period of stress (Sheveleva et al., 1997).

Throughout the *Rosaceae,* sorbitol is a major photosynthetic product translocated from leaves to roots and other sink tissues. Sorbitol is synthesized in source tissues from glucose-6-phosphate via the action of NADP dependent sorbitol-6-phosphate dehydrogenase (S6PDH) and a phosphatase (Loescher et al., 1982). In sink tissues, sorbitol phosphate is converted to fructose via the action of sorbitol dehydrogenase. Increased sorbitol in source tissues correlates with salt stress tolerance. For example, salt stressed Japanese pear, *Pyrus pyrifolia* (Burm. f.) Nakai, leaves showed increased sorbitol concentration, producing ^{14}C-sorbitol when incubated with ^{14}C-glucose. Glucose, fructose and sucrose showed no trace of ^{14}C, suggesting that under stress conditions sorbitol is the favored compatible solute in Japanese pear (Deguchi et al., 2002). In peach, *Prunus persica* (L.) Batsch, water stress transiently increases sorbitol content of the leaves but not the roots (Cui et al., 2004). Persimmons, *Diospyros kaki* (L.) f., transformed to express S6PDH from apple, *Malus* (P.) Mill., accumulated sorbitol where wild type did not. When placed under salt stress, the transformants maintained higher photosynthetic activity than untransformed controls (Gao et al., 2001).

The six carbon polyol, mannitol is found in over 100 plant species and is the most widely distributed hexitol in nature (Williamson et al., 2002). Mannitol is produced in celery, *Apium graveolens* L. by the conversion of fructose-6-phosphate to mannose-6-phosphate by phosphomannose isomerase. Two subsequent enzymes convert mannose-6-phosphate to mannitol (Stoop et al., 1996). In contrast, mannitol in bacteria is synthesized by the direct conversion of fructose-6-phosphate to mannitol-1-phosphate by mannitol-1-

phosphate dehydrogenase (mtlD). Mannitol-1-phosphate is then dephosphorylated by a phosphotase to form mannitol.

In addition to documented advantages in growth and metabolic efficiency (Stoop et al., 1996), mannitol and its catabolic enzyme, mannitol dehydrogenase (MTD), play an important role in salt tolerance (Pharr et al., 1995). MTD expression is tightly regulated in celery. In vegetatively growing plants and undifferentiated tissue, MTD expression is suppressed by high levels of sugar, osmotic stress and salt stress (Stoop et al., 1996). This tight control over MTD activity by celery is an advantage in terms of osmoregulation. The plant can use simple sugars under normal (non-stress) conditions while maintaining a pool of mannitol as a reserve carbohydrate and compatible solute (Stoop et al., 1996).

During salt or osmotic stress, mannitol can act as an osmolyte. In osmotically stressed celery, mannitol-6-phosphate dehydrogenase concentrations increase in the leaves while mannitol dehydrogenase levels drop in sink tissues. These changes are accompanied by consequent increases in mannitol concentrations in both sink and source tissues. (Williamson et al., 2002). It is proposed that mannitol forms a sphere of hydration with hydroxyl groups around proteins and membranes in a way similar to that speculated for other compatible solutes.

Numerous transformation experiments have aided in confirming the role of mannitol in osmotic and salt stress. Arabidopsis over-expressing the bacterial mannitol biosynthetic gene *mtlD* germinate better under salt stress than seeds of untransformed plants (Thomas et al., 1995). However, the proportion of transformed plants that germinated was not

significantly different in all cases from the proportion of germination of wild type Arabidopsis reported in this work.

Wheat, *Triticum aestivum* L., *mtlD* transformants showed increased height, fresh and dry weight over non-transformed plants grown under osmotic and salt stress. Callus from these transformants grown on PEG 8000 (-1.0 mPa) and NaCl fortified (100mM) media showed significantly increased mass over control (Abebe et al., 2003).

Tobacco *mtlD* transformants grown under salt stress maintained dry weight while the dry weight of untransformed plants dropped by 44%. Fully expanded leaves on these plants were able to osmotically adjust when exposed to salt stress where leaves from non-transformed plants were unable to do so. In addition, transgenic tobacco synthesizing chloroplast-targeted mtlD exhibited increased oxidative stress tolerance, suggesting that, in addition to osmoregulation, mannitol is able to supplement endogenous free radical scavenging mechanisms (Shen et al., 1997).

Approaches to Increasing Production in Saline Environments

Advanced Breeding

While traditional breeding remains a viable approach for many traits, its usefulness in developing salt tolerant plants is limited by the complex nature of salt and osmotic stress tolerance. As described above, traditional breeding has been slow in developing a salt tolerant tomato. However, it is expected that, through the use of quantitative trait loci (QTLs, specific loci where allelic variation is associated with variation in a quantitative trait)

and marker assisted selection, species and varieties showing salt tolerance at various developmental stages can be selected for breeding (Foolad, 2004).

Other work with tomato has used wide-hybrid pollen tube germination techniques. This technique allows the direct crossing of two otherwise incompatible but related species. Several wild salt tolerant species have been selected based on the presence of the *HV-A1* gene, a member of the LEA family. The successful transfer of this gene between the wild relative and commercial varieties has been successful, and progeny of these crosses showed improved tolerance to saline conditions (Chen et al., 2004).

Tomato is not the only vegetable with salt tolerant relatives. Members of the genus *Phaseolus* exhibit diverse salinity tolerance, with 23 tolerant and 18 moderately tolerant taxa identified. Use of QTLs (Bayuelo-Jimenez et al., 2002) and marker assisted selection should speed up development of new cultivars. In citrus there is also promise of using these techniques to develop salt (Mademba-Sy et al., 2003) and aluminum tolerant cultivars (Samac and Tesfaye, 2003).

Salt tolerance of field crops also can be improved using wide-hybrid techniques. *Porteresia coarctata* is a highly salt tolerant relative of rice growing naturally in coastal regions of India and Bangladesh. Wide hybridization or bridge species breeding will be useful in transferring some of this tolerance to domesticated rice cultivars (Latha et al., 2004). The observed advantages of compatible solute synthesizing plants have made the genes responsible for their synthesis attractive targets for both advanced breeding techniques and transgenic work.

Transgenics

In contrast to even wide-hybrid crosses, transgenic approaches allow the introduction of potentially useful genes from an almost unlimited variety of sources. For instance the bacterial mannitol biosynthetic gene *mtlD* has been transformed into a number of plants including tobacco, wheat and Arabidopsis.

Wheat *mtlD* transformed plants and calli were able to accumulate mannitol as an osmoprotectant during salt and osmotic stress (Abebe et al., 2003). Tobacco *mtlD* transformants showed improved resistance to salt stress and an ability to adjust water potential not seen in wild type (Tarczynski et al., 1993). Also, when the same gene is targeted to tobacco chloroplasts, the plants tolerate a greater degree of photooxidative stress (Shen et al., 1997).

Other genes under investigation as targets for transgenic plant improvement include transporters and numerous enzymes. Transgenic *BADH-1* tomatoes maintained water potential under osmotic stress where wild type did not (Moghaieb et al., 2000). Barley transformed with the wheat malate transporter gene (*ALMT1*) showed improved aluminum tolerance (Delhaize et al., 2004). Transgenic Arabidopsis expressing proteins for increased Ca^{2+} capturing capacity in the lumen of intracellular compartments grew well on calcium depleted media, suggesting greater stress response ability due to higher levels of available Ca^{2+} (Wyatt et al., 2002).

Management Practices

The development of tolerant cultivars will help mitigate short-term productivity losses due to salt stress, but will be fruitless if current shortsighted agricultural practices are continued. Unchanged practices promise the eventual loss of millions of hectares of currently productive land.

An immediate improvement could come from changing irrigation techniques to favor those conserving water. For example, drip irrigation uses far less water than overhead irrigation. Less water use means less salt accumulation. Also, reducing demand would slow or stop the incursion of seawater into freshwater aquifers.

In the long term, selecting crops appropriate to the growing conditions could slow or even halt soil salinization due to human activity. There are agricultural species, such as date palms, that grow well in arid areas with slightly saline soils. To continue growing irrigation intensive crops, such as strawberries, in arid regions only serves to accelerate the problem, so switching to those crops requiring less water would help.

There are also possibilities for reversing the trend of salt buildup by using bioremediation. Many plants, particularly grasses, have anatomical structures designed to store concentrated salt. For example within the forage and turf grass subfamily, *Chloridoideae* several members take up and store salts in special glands. These sorts of plants could be used to reverse salinity problems, if grown and harvested on an ongoing basis. All of these management approaches combined, would stand a very good chance at reversing a growing problem.

Opportunities for Further Study

Stage of Development and Salt Tolerance.

Known salt tolerant plants have been examined in some detail to develop an understanding of osmotic and salt tolerance mechanisms. These studies are conducted with an eye toward developing improved plant cultivars. Work with Japanese pear has shown that sorbitol is an important salt stress related osmolyte in this species' leaves (Deguchi et al., 2002). Researchers studying the osmotic stress tolerance of *Mesembryanthemum crystallinum* L. found that adult plants accumulate large amounts of ononitol in the leaves, enabling the protection of photosynthesis and sequestration of sodium (Nelson et al., 1998).

Celery plants and cell cultures accumulate mannitol under salt stress, enabling the plant to maintain dry weight gain despite conditions (Pharr et al., 1995). Each of these findings is useful in understanding mechanisms of salt tolerance. It is well known though, that tolerance at one stage of development may not be expressed at another. Stress responses are regulated throughout the plant, and development is tissue specific, so studying isolated life stages perhaps reveals only part of the story (Borsani et al., 2003).

Carbohydrates and Salt Tolerance.

Another line of investigation of salt tolerance mechanisms involves carbohydrate content changes in salt or osmotically conditioned plants. Sucrose for instance, plays a role in enabling *Craterostigma plantagineum* Hochst to survive nearly complete desiccation (Norwood et al., 2000). Also, a drought tolerant cultivar of chickpea, *Cicer arietinum* L., shows a higher level of α-amylase activity in cotyledons of germinating seeds than a drought sensitive cultivar (Gupta et al., 1993) suggesting that soluble sugars might play a role in that

drought tolerance. However, it is important to note that many species employed for investigating salt tolerance express the genes for salt tolerance only after a period of acclimation (Borsani et al., 2003). For example, *Aspergillus ochraceus* K. Wilh. accumulates high levels of mannitol in its conidia only when grown under osmotic or salt stress (Ramos et al., 1999), and osmotically stressed germinating wheat seeds accumulate high concentrations of free sugars (Thind, 1991). Osmotically stressed seeds, which are then dehydrated before radicle elongation, germinate more uniformly and vigorously upon re-imbibition. The process, called seed priming, increases enzyme activity, RNA transcript accumulation and membrane repair. However, little is published on carbohydrate changes in osmotically stressed seeds.

Our understanding of the mechanisms of salt and osmotic stress tolerance in developed tissues continues to grow. An area unexamined, but of equal importance in the complete life cycle of plants, is understanding mechanisms of salt and osmotic stress tolerance during seed germination. Many researchers have investigated the role of carbohydrates in the stress response of adult plants and fully developed tissues. However, the changes in carbohydrate content of germinating seed under salt and osmotic stress has been much less investigated. We have chosen to investigate the germination and development of salt tolerant and sensitive taxa. Our efforts will, hopefully, add to the understanding of plant development and stress response during this critical point in the plant's life cycle.

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Chapter 2

Carbohydrate content and root growth in seeds germinated under salt stress: implications for seed conditioning

This chapter was prepared in the style of the Journal of the American Society of Horticultural Science and will be submitted for review

Abstract. Because salt stress can reduce photosynthesis and yields as well as reduce crop value by damaging appearance, field, container, and greenhouse producers must manage salinity carefully to assure a profitable crop. Understanding plant responses to salt stress is key in managing this problem. A common protective response to salt stress is osmoregulation and/or osmoprotection via the accumulation of carbohydrates or compatible solutes such as the sugar alcohol mannitol. Various protective roles for these compatible solutes have been well documented in plants, cell cultures and maturing seed. Less information, however, is available on possible impacts of these compounds on protection against salt stress during the germination process itself. To specifically assess potential roles for sugars and sugar alcohols on germination, growth and carbohydrate metabolism, we selected seed from known mannitol and non-mannitol accumulating salt tolerant taxa and seed from known salt sensitive non-mannitol accumulating taxa, and germinated them in the presence of increasing concentrations of sodium chloride.

Seed of both salt tolerant and sensitive taxa showed varying degrees of decreased radicle elongation as salt stress increased. Surprisingly, seed from taxa known to accumulate mannitol did not germinate better in saline environments than did seed from non-mannitol producing taxa. In fact, seed from non-mannitol taxa described as salt sensitive had somewhat higher germination and better growth under salt stress than seed of salt tolerant mannitol-containing plants. Analysis of soluble carbohydrates in seeds and seedlings incubated in the presence of increasing salt showed no overall correlation between mannitol content and germination or growth. However, accumulations of low molecular weight carbohydrates, especially sucrose, were observed at high levels of salt stress. Interestingly, we observed this increase most often after concentrations of salt were reached that totally inhibited germination, suggesting a potential role for carbohydrates in conditioning of seed during salt stress.

Saline soils are common in semi arid and arid areas of the world. Worldwide it is estimated that 33% of irrigated lands are affected by salinization and that land passing out of irrigation due to salinization exceeds land becoming available (see Marschner, 1998). Agricultural and human activity accelerates this process. Saline soils can develop through evaporative concentration of salts in fields irrigated with brackish water or by incursion of seawater into freshwater aquifers due to excess agricultural and urban demand.

Damage to plants from saline soils results from both specific ion effects and osmotic stress. For instance, sodium and other ions can disrupt proteins by passing through the sphere of hydration and disrupting hydrogen bonds and Van der Waals interactions resulting in de-naturation and the loss of protein function. Sodium can also bind to photosystem two (PSII) and irreversibly block the oxygen evolving machinery, thus inhibiting electron transfer from water. (Allakhverdiev et al., 2000). The ensuing generation of reactive oxygen species (ROS) from the inhibited PSII can then result in destruction of associated proteins and membranes. Evidence of such damage appears as necrotic lesions on leaves. Osmotic stress itself can increase solute concentration due to loss of water or localized influx of ions. These changes in mineral and ionic balance can affect membrane integrity, photosynthetic efficiency, protein conformation and enzyme function. For example, increased concentrations of magnesium (Mg^{2+}), an essential electron-transfer mediating component of chlorophyll, inhibits photosynthesis in water stressed sunflower (Rao et al., 1987).

Plant cellular resistance mechanisms to these stresses are diverse and include increases in concentrations of proteins involved in water transport, ion sequestration and secretion (Baiges et al., 2002; Figueras et al., 2004), as well as increases in osmolytes and/or compatible solutes

derived from amino acids, sulfonium and ammonium compounds, and carbohydrates (Bray et al., 2000). Compatible solutes are so called due to their ability to accumulate to high concentrations without disrupting cellular processes. Osmolytes specifically balance differences in osmotic potential between the cytosol, apoplast and tonoplast in response to ionic and osmotic disruption. Compatible solutes are also thought to maintain a sphere of hydration around proteins or membranes to allow continued function under stress conditions. Carbohydrate derived osmolytes and compatible solutes include sugars such as sucrose, glucose and fructose, polymers like the raffinose family oligosaccharides (RFOs), and polyols such as pinitol, ononitol, and mannitol.

Sucrose, fructose, and glucose all act as osmolytes, and can be quickly generated from polymeric carbohydrates in response to osmotic stress (Levitt, 1980). A related process is seen during germination when stored RFOs are converted to sucrose or monomeric sugars (Peterbauer and Richter, 2001). Another example of this type of conversion in response to osmotic stress is seen in the resurrection plant *Craterostigma plantagineum* Hochst. Well watered plants accumulate the eight carbon carbohydrate 2-octulose, but as osmotic stress increases octulose is converted to sucrose (Norwood et al., 2000). This type of carbohydrate flux appears to be a common phenomenon in desiccation tolerant seeds (Peterbauer and Richter, 2001; Prado et al., 2000). Raffinose oligosaccharides are a common component of seed storage and are believed to form, with sucrose, a vitrified environment that stabilizes desiccated seed membranes. RFOs accumulate during seed maturation and are converted rapidly during germination to monomeric sugars (Obendorf, 1997).

The roles of sugar alcohols in salt tolerance are well documented (e.g. see Williamson et al., 2002). Studies of vegetatively growing plants and tissue cultures have shown mannitol accumulates in response to salt stress. In osmotically stressed celery plants the activity of mannitol synthesizing enzymes increase in the leaves while sink tissues show a decrease in

mannitol dehydrogenase (MTD) activity. These changes are accompanied by consequent increases in mannitol concentrations in both sink and source tissues (Stoop et al., 1996). Separate cell populations grown such that they have equimolar concentrations of sugars or mannitol do not exhibit the same degree of salt stress tolerance (Pharr, 1995). Mannitol accumulating cells maintain a higher growth rate, suggesting mannitol functions as an osmoprotectant rather than simply being an osmolyte. A less studied but promising role for polyols is that of free radical quenchers. Many have been shown to counter the damaging effects of ROS both in vivo and in vitro (see Williamson et al., 2002). For instance, Shen et al. (1997) showed that tobacco plants expressing chloroplastic mannitol dehydrogenase could withstand a greater degree of oxidative stress than wild type and had an improved ability to scavenge hydroxyl radicals.

From the above, it is clear that carbohydrate derived compatible solutes play important roles in salt and osmotic stress tolerance. Studies of extant mechanisms demonstrate that simple sugars can stabilize membranes in desiccated *Craterostigma plantagineum* Hochst. Transgenic experiments have likewise demonstrated mannitol's role in tolerance to ROS and salt in plants and cell cultures. Left unexamined is the question of how carbohydrates in seed (both pre-existing and those accumulating during germination) affect germination and root elongation under salt stress. As salt tolerant plants presumably germinate in high salt environments, we designed experiments to assess germination of a variety of plants under salt stress. Because mannitol is known to have roles in salt tolerance, we selected salt tolerant taxa both with and without mannitol in dry seed (celery and cabbage respectively) as well as salt sensitive non-mannitol producing taxa (tobacco and Arabidopsis). Seeds were germinated in the presence of increasing concentrations of salt, and daily measurements of root elongation and germination were made, together with analysis of seed and harvested seedling carbohydrate content. Our results suggest a possible role for carbohydrate metabolism in the process of seed priming.

Materials and Methods

Plant Material. Plant varieties and seed sources were: Salt sensitive taxa, *Arabidopsis thaliana* (L.) Heynh 'Columbia' and *Nicotiana tabacum* L. K326, donation by Dr. Steve Clouse; a non-mannitol synthesizing salt tolerant plant, *Brassica oleracea* L. *capita* 'Golden Acre' Wyatt-Quarles Seed Company, Garner, N.C., and two salt tolerant mannitol synthesizing taxa, *Apium graveolens* L. *dulce* (P. Mill.) DC 'Florida' (Celery 1), Asgrow Seed Company, Kalamazoo, Mich., *Apium graveolens* L. *dulce* (P. Mill.) DC 'Ventura' (Celery 2), Johnny's Select Seeds, Winslow, Maine. Seeds were sterilized in 70% ethanol with a gentle rocking motion for 5 min then rinsed with sterile water. Next a 30% bleach solution containing 0.05% Tween was added. Seeds were gently rocked for 10 min then rinsed 5 times with sterile distilled water. Sterilized seeds were suspended in sterile 0.01% agar then pipetted onto growth media in 15cm polystyrene petri plates at equidistant locations, 50 seeds per plate. Growth media contained $9g \cdot L^{-1}$ of Phytagel (Sigma Aldrich Inc., Milwaukee., P8169) and $4.4g \cdot L^{-1}$ MS salts plus vitamins (Caisson Laboratories Inc., Rexburg, Idaho, MSP002) with a pH of 5.8 with the indicated amount of sodium chloride added. Plates were incubated vertically at 22°C under a 12h/12h photoperiod at $150\mu E \cdot m^{-2} \cdot s^{-1}$. Seedlings were grown until the first lateral roots began to form or the plants began to touch.

Data Collection. Germination in this study was defined as a radicle elongation greater than the diameter of the seed. No lengths were recorded unless they were greater than this measure. Therefore, a recording for length automatically records germination. Each plate was

photographed daily using a digital camera. The photographs were brought to scale using photo-editing software (Knoll and Hamburg, 2001), and a 1mm grid was superimposed over the image. Root length was recorded for each seedling on each plate.

Carbohydrate Analyses

Extraction of Soluble Sugars. Harvested seeds and seedlings for all replications in a given treatment were frozen in liquid nitrogen and pulverized. Of this material up to 200mg was added to 3 mL of 80% ethanol and ground in a mortar and pestle. The solution was transferred to a 15mL centrifuge tube, and the process was repeated twice, producing a total of 9mL. The solution was centrifuged at 750 x g for 15 minutes to pellet debris, and the supernatant fluids evaporated using a rotary evaporator (Buchler, Evapo-Mix. Fort Lee, NJ). After evaporation the remaining solute was dried at 60°C for 12 hours in a drying oven and resuspended in 1mL of deionized distilled water. Before HPLC analysis samples were centrifuged at 10,000 x g in a microcentrofuge for 5 minutes to remove any remaining insoluble material.

Analysis of Soluble Sugars. Carbohydrate content of the samples was analyzed using a CarboPac PA-1 colunm (Dionex Inc., Sunnyvale, Calif., Cat No. 035391), 250 mm length, 4 mm i.d., fitted with a CarboPac PA-1 guard column (Dionex Cat No. 043096). The column oven was set to 30°C. The mobile phase used 200mM NaOH sparged with helium for 2 hours. A 25µL sample loop was used and 10µL of water was used as the injector carrier solvent. The flow rate was set to 1.0mL·min^{-1} (isocratic) using a Dionex GS50 gradient pump. The detector was a Dionex PAD (Pulsed Amperometric) set to 100 nC. Samples were filtered through Dionex OnGuard II H

columns to remove free amino acid interference. The samples were run with dilutions of standards to construct five point curves fitted to zero.

Statistical Analysis. Experiments were set up in a randomized complete block design with five blocks. Significance of percent germination and root length measurements for each taxon was tested using the general linear model (GLM) with partial sum of squares as analyzed by a commercial software package (SAS Institute, Cary, N.C.).

Non-linear regression using the Weibull model (Dias, 2001) was performed using the NLIN procedure of SAS v8 (SAS Institute) on the germination data from all blocks to test statistical significance of the germination rate, scale, distribution and response curve. The regression model used was:

$$Y = a\left(1 - e^{-\left\{\left[\frac{(X-l)}{k}\right]^{c}\right\}}\right)$$

where Y is the proportion of germinated seed on day X, a, is an estimate of overall germination proportion, *l* is an estimate of the latest day where germination was 0 and is treated as a constant here derived from direct observation, *k* (days) is a scale parameter with $l + k$ estimating the time at which proportion of germination is 0.63. The dimensionless parameter *c* estimates the symmetry of the germination distribution away from the normal curve ($c > 3.60$ negative asymmetry, $c < 3.26$ positive, and $3.26 \leq c \leq 3.60$ symmetrical). The resulting Weibull estimates for each block were tested for significance using GLM. The Weibull estimates were used to calculate G_D values (Days to a proportion of germination), which were tested for significance using general linear models similar to those for germination and root length.

Significance of differences between taxa for a specific treatment was established using least squares means with a combined error term of block and variety. Differences between treatments for specific taxa were also tested with the least squares means method and an error term of block and treatment.

Results

To ensure the sterilization procedure used in these experiments did not adversely affect seed viability, sterilized seeds were first test-germinated on moist paper towels and germination compared to germination of non-sterilized seed. As shown in Table 1, germination was not seriously affected by sterilization. To assess the effect of salt stress on germination and growth, seed of salt-tolerant mannitol-synthesizing and non-mannitol-synthesizing taxa and salt sensitive non-mannitol synthesizing taxa were incubated on media with increasing amounts of added sodium chloride. Daily measurements of root growth and germination were made, and possible correlations with carbohydrate content of harvested seedlings and dry seed were examined.

Effects of Salt Stress on Radicle Elongation. Root length data is summarized in Table 2 as a percentage of control (no added salt) and absolute values in mm. Each taxon showed a strong immediate treatment effect with the exception of cabbage, which demonstrated a remarkable ability to continue growth at only slightly reduced levels in 200mM added NaCl. Tobacco and Arabidopsis, selected for their reported salt sensitivity surprisingly continued growth at a low level in 200mM added NaCl. In contrast, the two varieties of celery, selected for their reported salt tolerance, completely stopped elongating at around 100mM to 150mM added NaCl. Of the

five taxa studied, only cabbage and tobacco elongated significantly in NaCl treatments higher than 100mM.

Effects of Salt Stress on Germination. Biologically relevant analysis of germination requires not only assessment of the cumulative proportion of germinated seeds, but must also include time to onset of germination, and the shift in germination distribution. These assessments can then be used to calculate the delay in germination expressed as days to reach a particular proportion of germinated seeds (G_D). The Weibull method used to describe phytotoxic effects during germination has been shown a superior tool for this type of analysis (e.g. Dias, 2001; Verdu and Mas, 2004). Weibull estimates are used in conjunction with actual proportion of germinated seeds for assessing overall treatment effects.

Differences in germination between salt treatments for each species (Fig. 1 and Table 3) are apparent not only in the depression of proportion of germinated seeds (P_g), but also in delays in the onset of germination (k) and distribution of germination (c). The proportion of germination in both celery cultivars was strongly reduced by 50mM added NaCl. P_g dropped 20% in celery 1 and 40% in celery 2. At 100mM added NaCl P_g was reduced 63% and 95% respectively, while in the reportedly salt sensitive species Arabidopsis and tobacco, germination decreased only 12% and 11% respectively. In fact, Arabidopsis and tobacco maintained similar proportions of germinated seeds compared to cabbage up to 150mM added salt. Cabbage showed no significant difference in proportion of germinated seeds until the 200mM added salt treatment. Both, Arabidopsis and tobacco showed a statistically greater ability to germinate at 50mM and 100mM added salt compared to both celery varieties, and germination was not greatly diminished until the 150mM added salt treatment.

Although both Arabidopsis and tobacco maintained statistically similar degrees of "final" germination in all treatments, tobacco had a much greater delay in the onset of germination in response to salt (Fig. 1). Delays in germination caused by treatment are expressed here as days to a given proportion of germination (G_D), and as a days to onset of germination (*k*). For example, examining the difference in G_{50} between the control and +50mM treatment, we see that cabbage was least affected, followed by Arabidopsis whose G_{50} was delayed by 1.3 days. The celery cultivars were delayed approximately 1.9 days, and tobacco 3.3 days. Onset of germination estimates (*k*) showed a similar pattern within this treatment range.

Assessment of Carbohydrate Content. To see if there was any apparent correlation with ability to germinate, we assessed changes in type and amount of carbohydrate in untreated seeds and seedlings incubated under increasing salt stress. Carbohydrates were extracted from salt stressed seedlings and dry seeds and analyzed using pulsed amperometric HPLC. The results are summarized in Table 4 and Fig. 2.

At first glance, there seemed to be no consistent pattern between the ability to germinate in elevated salt and carbohydrate content. For instance, although the most tolerant species, cabbage, had high carbohydrate concentrations, the next most tolerant, Arabidopsis, had the lowest concentrations (Table 4). Cabbage and Arabidopsis, however, were the only taxa that contained significant amounts of raffinose and/or stachyose. Interestingly, although the two mannitol containing celery varieties were among the most salt sensitive, mannitol content (per seedling) initially did increase at increasing concentrations of salt (e.g. 50 mM added salt). As germination declined at higher salt concentrations (greater than 100 mM) (Fig. 2) sucrose became the dominant soluble carbohydrate. The only "global" correlation appeared to be that, in all taxa with the possible exception of cabbage, sucrose increased dramatically in seedlings in the lowest

salt concentration that completely inhibited root elongation. Although seeds had ceased germination at this point, the amount of sucrose in these seedlings was two to eight times higher than amounts of sucrose in the dry seeds. For cabbage, the amount of sucrose also increased somewhat at salt concentrations in excess of 150 mM added salt, but seedlings were still growing at the highest salt concentrations used in these experiments (300 mM).

Discussion

Salt tolerance in plants has been the subject of voluminous research. For a variety of reasons, however, research has focused primarily on tolerance in postgermination seedlings and mature plants. Less information exists on the ability of seed to germinate under saline conditions. To assess possible contributions of seed or seedling carbohydrate content to the ability of seeds to germinate under saline conditions, we evaluated seeds from plants that in studies of vegetatively growing plants and cell cultures show a wide range of salt tolerance and seed carbohydrate composition. For instance, mannitol-synthesizing plants such as celery have been shown to grow well under osmotic and salt stress (Pharr et al., 1995), as do RFO-accumulating plants such as cabbage (Minorski, 2003). Conversely, the non-mannitol plants Arabidopsis and tobacco are commonly reported to be salt sensitive (Thomas et al., 1995; Karakas et al.,, 1997). When seeds from these species were imbibed and germinated in the presence of NaCl, however, quite a different picture emerged. While cabbage germinated and grew well under salt stress, the putatively salt sensitive species Arabidopsis and tobacco germinated and grew better under salt stress than did two varieties of celery. Further, there seemed to be no consistent pattern between ability to germinate in elevated salt and carbohydrate content. These results suggest that not only do different plant species use different mechanisms to provide salt tolerance during germination, but that even where the same mechanism is employed, it may not provide identical protection.

For instance, the most tolerant species, cabbage, had by far the highest seed and seedling carbohydrate content, but the next most tolerant, Arabidopsis, had the lowest (Fig. 2). Cabbage and Arabidopsis seeds did, however, both contain significant amounts of raffinose and/or stachyose, which have been associated with both salt and osmotic stress tolerance. Further, protective mechanisms that might be effective at lower stress levels might be less effective at higher levels. For instance, although the mannitol-containing celery varieties were ultimately among the most salt sensitive, mannitol content did initially increase in response to increasing salt. At higher salt treatments, however, mannitol content decreased. The broadest correlation observed, however, was a large increase in sucrose in seedlings at the lowest salt concentration that completely inhibited growth. Although seeds failed to germinate at this point, they were still biochemically active as indicated by the fact that amounts of sucrose were two to eight times higher than in dry seeds.

The observed differences in germination between tolerant and sensitive species reflect a greater salt sensitivity than expected. The salt sensitivity of the normally tolerant species seemed counterintuitive, because plants such as celery that have evolved in saline environments must also be able to germinate in the presence of salt. On reflection, two possible explanations for these results suggest themselves.

Environmental conditions during seed development are known to affect the physiology and biochemistry of the mature seed. For example, in sand plain lupine, *Lupinus angustifolius* L., differences in diurnal temperature regimen result in differences in the coat permeability of the mature seed (Koller, 1972). Also, Von Abrams and Hand (1956) established that *Rosa* L. seeds harvested from greenhouse plants had a lower dormancy requirement than those harvested from field grown plants. This type of developmental response is not limited to plants, but is conserved across kingdoms. For example, the fungus *Aspergillus ochraceus* K. Wilh. accumulates high

concentrations of mannitol in conidia when grown under osmotic or salt stresses (Ramos et al., 1999). Similarly, one might expect a plant adapted to a saline environment, such as celery, to "precondition" seed to germinate under salt stress. The commercial seed used in this study was likely from plants grown under non-saline conditions, and thus would not be preconditioned to grow under salt stress.

The early events of seed germination are devoted to the repair and synthesis of needed structures and enzymes, and mobilization of stored food reserves. Under non-saline conditions during the first hours post-imbibition membranes are leaky and small molecules such as ions, sugars and amino acids leach into the surrounding medium or seed tissues. Subsequently, membranes and organelles are repaired and protein synthesis begins. In some cereal grains GA produced by the embryo induces synthesis of α-amylase, starch is hydrolyzed and stored carbohydrates are mobilization for embryo growth (Bewley, 1997). At the same time, proteolysis of storage proteins by amino- and/or endopeptidases allows mobilization of amino acids for new protein synthesis or nitrogen scavenging. Finally, lipids hydrolyzed by lipases yield fatty acids and glycerol. Carbon from these compounds moves from the oleosome to the glyoxysome then to the mitochondrion and finally to the cytosol for gluconeogenesis.

The process of germination is surprisingly robust, and many seeds can complete the early requisite metabolic events of germination in a highly saline environment without actually germinating, and then be redesiccated without damage. This is the basis of osmotic priming, a technique widely used to improve germination uniformity and speed by first imbibing seed in a high osmotic potential solution (Parera and Cantliffe, 1994). Seeds complete the early metabolic events necessary for germination where membranes are repaired and enzymes required for growth are synthesized (Hong et al., 1997; Singh and Kumar, 1992; Thind, 1991). Germination

does not proceed beyond this point, however, (i.e. there is no radicle elongation) due to the extreme osmotic stress. Seeds so treated can then be dehydrated without damage, and when reimbibed, germinate more rapidly and uniformly than unprimed seed.

The increasing lag time before the onset of germination in increasing salt, the suppression of germination and the large accumulation of sucrose in seed in high salt (Figs. 1 and 2), all appear consistent with what is known of seed priming (Hong et al., 1997; Singh and Kumar, 1992; Thind, 1991). These results might suggest that, in addition to conditioning of seed by the parent plant, osmotic priming might also be a normal mode of germination for plants such as celery that are adapted to growth in saline environments. Seed imbibed in the presence of inhibiting concentrations of salt could undergo the first metabolic events of germination and become primed for subsequent rapid growth when salt concentrations decreased (e.g. after a rainstorm). Once past germination, previously described salt tolerance mechanisms (i.e. mannitol accumulation) could predominate.

Plants employ a variety of adaptive mechanisms in response to salt stress. Our results suggest that these mechanisms not only vary among species, but may also be employed with different effectiveness at various developmental stages. For instance, the accumulation of mannitol is a very potent protective mechanism against salt stress in vegetatively growing celery. However, in germinating seed, sucrose and not mannitol appeared to be the primary compatible solute under extreme salt stress. In addition, our results suggest that different mechanisms may be mobilized independently or in combination, depending on the severity of the stress. For example, mannitol accumulated in mildly salt stressed celery seedlings, but as the stress increased sucrose accumulation became the dominant response. These results are consistent with earlier work such as that of Dumbroff et al. (1974) who showed that tomato is far less salt tolerant at earlier stages of development than at the flowering or fruiting stage. A clearer picture of the salt

stress mechanisms employed through the entire life cycle of a plant allows breeders to identify additional sources of germplasm for use in breeding new cultivars. Because developing salt tolerant cultivars of important crop plants will be necessary to maintain productivity on saline soils, continued research on salt and osmotic tolerance mechanisms is critical.

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Table 1: Effect of sterilization on seed germination. Unsterilized and sterilized stock seed (150 – 200 seeds) were germinated on moist paper towels after imbibition. Results shown are percent of germinated seed in each treatment by day 10).

Taxa	Unsterilized	Sterilized
Arabidopsis	96%	94%
Cabbage	74%	63%
Celery 1	79%	80%
Tobacco	92%	94%
Celery 2	86%	79%

Table 2: Changes in root elongation under increasing concentrations of sodium chloride. Root lengths were recorded daily for each taxon and treatment combination. Seedlings were grown on media containing increasing concentrations of NaCl. Differences in length at the end of the growth period are expressed here as absolute values ± standard error followed by percent of control.

	Concentration of Added NaCl *Root Length (mm)*					
	+0mM	+50mM	+100mM	+150mM	+200mM	+300mM
Arabidopsis	16 ± 0.9 a[z]	11 ± 0.9 (67%) b	6 ± 0.9 (37%) c	1 ± 0.9 (8%) c	1 ± 0.9 (7%) c	0.0
Cabbage	29 ± 1.4 a	26 ± 1.4 (86%) a	28 ± 1.4 (94%) a	19 ± 1.4 (67%) b	14 ± 1.4 (43%) b	2 ± 1.4 (7%) b
Celery1	14 ± 0.5 a	10 ± 0.5 (70%) b	6 ± 0.5 (44%) c	1 ± 0.5 (8%) c	0.0	0.0
Tobacco	7 ± 0.5 a	6 ± 0.5 (88%) a	3 ± 0.5 (39%) b	1 ± 0.5 (15%) b	1 ± 0.5 (15%) b	0.0
Celery2	14 ± 0.4 a	9 ± 0.4 (61%) b	3 ± 0.4 (27%) c	0.0	0.0	0.0

[z]In the same row means followed by a different letter (a, b, c, d) are significant to the $p < 0.005$ level according to GLM.

Table 3. Analysis of seed germination under increasing concentrations of salt. Seed of salt tolerant (cabbage and celery) and sensitive (tobacco and Arabidopsis) taxa were germinated on media containing increasing amounts of added NaCl. Numbers of germinated seeds were recorded daily for each repetition, treatment and taxa, and statistical analyses performed using Weibull analysis as described in Materials and Methods. Data shown are proportion of seed geminated (Pg), days to reach a given proportion of germination (G_D), and estimates of maximum germination proportion (***a***), shift in germination distribution symmetry (***c***, unitless), and changes in the sca1e of germination (***k***, days). Weibull parameter estimates were derived for each block using existing germination data and were tested for significance using GLM. Data are means ± SE. G_D values for each b1ock were calculated using the Weibull parameters shown and tested for significance using GLM. The germination rate = *1/k*.

		+0mM	+50mM	+100mM	+150mM	+200mM
Arabidopsis						
	Pg	0.93 ± 0.05 a[z]	0.89 ± 0.05 a	0.82 ± 0.05 a	0.46 ± 0.05 b	0.28 ± 0.05 b
	G_{10}	1.3 ± 0.24 a[y]	2.2 ± 0.24 b	3.1 ± 0.24 c	5.2 ± 0.24 d	0.0
	G_{25}	1.6 ± 0.25 a	2.7 ± 0.25 b	3.8 ± 0.25 c	6.0 ± 0.25 d	0.0
	G_{50}	1.9 ± 0.26 a	3.2 ± 0.26 b	4.5 ± 0.26 c	6.7 ± 0.26 d	0.0
	a	0.94 ± 0.03 a[x]	0.87 ± 0.03 a	0.77 ± 0.03 b	0.46 ± 0.03 c	0.00
	c	0.76 ± 0.17 a	1.42 ± 0.17 b	1.97 ± 0.17 c	3.21 ± 0.17 d	0.00
	k	1.7 ± 0.20 a	3.2 ± 0.20 b	4.3 ± 0.20 c	5.5 ± 0.20 d	0.0
Celery 1						
	Pg	0.80 ± 0.03 a	0.64 ± 0.03 b	0.29 ± 0.03 c	0.00	0.00
	G_{10}	3.7 ± 0.19 a	5.2 ± 0.19 b	7.2 ± 0.19 c	0.0	0.0
	G_{25}	3.8 ± 0.21 a	5.4 ± 0.21 b	7.5 ± 0.21 c	0.0	0.0
	G_{50}	4.0 ± 0.22 a	5.7 ± 0.22 b	7.8 ± 0.22 c	0.0	0.0
	a	0.79 ± 0.03 a	0.62 ± 0.03 a	0.29 ± 0.03 c	0.00	0.00
	c	2.44 ± 0.27 a	2.97 ± 0.27 b	4.70 ± 0.27 c	0.00	0.00
	k	3.0 ± 0.19 a	4.6 ± 0.19 b	6.4 ± 0.19 c	0.0	0.0
Cabbage						
	Pg	0.63 ± 0.04 a	0.60 ± 0.04 a	0.56 ± 0.04 a	0.50 ± 0.04 a	0.46 ± 0.04 a
	G_{10}	1.2 ± 0.14 a	1.3 ± 0.14 a	0.0	0.0	0.0
	G_{25}	1.3 ± 0.17 a	1.5 ± 0.17 a	0.0	0.0	0.0
	G_{50}	1.6 ± 0.19 a	1.6 ± 0.19 a	0.0	0.0	0.0
	a	0.81 ± 0.08 a	0.83 ± 0.08 a	0.00	0.00	0.00
	c	1.58 ± 0.21 a	1.30 ± 0.21 a	0.00	0.00	0.00
	k	3.5 ± 1.46 a	6.6 ± 1.46 a	0.0	0.0	0.0
Celery 2						
	Pg	0.79 ± 0.03 a	0.47 ± 0.03 b	0.00	0.00	0.00
	G_{10}	4.6 ± 0.12 a	6.0 ± 0.12 b	0.0	0.0	0.0
	G_{25}	4.7 ± 0.13 a	6.2 ± 0.13 b	0.0	0.0	0.0
	G_{50}	4.9 ± 0.14 a	6.4 ± 0.14 a	0.0	0.0	0.0
	a	0.79 ± 0.03 a	0.62 ± 0.03 b	0.00	0.00	0.00
	c	2.47 ± 0.27 a	2.97 ± 0.27 a	0.00	0.00	0.00
	k	3.0 ± 0.19 a	4.6 ± 0.19 b	0.0	0.0	0.0
Tobacco						
	Pg	0.94 ± 0.04 a	0.94 ± 0.04 a	0.84 ± 0.04 a	0.42 ± 0.04 b	0.18 ± 0.04 c
	G_{10}	1.8 ± 0.31 a	4.5 ± 0.31 b	5.7 ± 0.31 c	8.9 ± 0.31 d	0.0
	G_{25}	2.2 ± 0.34 a	4.9 ± 0.34 b	6.5 ± 0.34 c	9.7 ± 0.34 d	0.0
	G_{50}	2.7 ± 0.37 a	5.3 ± 0.37 b	7.2 ± 0.37 c	10.3 ± 0.37 d	0.0
	a	0.96 ± 0.02 a	0.92 ± 0.02 a	0.85 ± 0.02 b	0.44 ± 0.02 c	0.00
	c	2.08 ± 0.51 a	6.99 ± 0.51 b	5.15 ± 0.51 c	8.14 ± 0.51 d	0.00
	k	2.7 ± 0.29 a	4.9 ± 0.29 b	7.2 ± 0.29 c	9.6 ± 0.29 d	0.0

[z]In the same row, means foll1owed by different letter (e.g. a, b, or c) are significantly different at $p < 0.0001$. [y]G_D values are ± SE and are

significant at $p < 0.05$ in the same row if fol1owed by different letters. [x]Data for the parameters ***a, c*** and ***k*** are indicated ± SE, and are significant at $p < 0.05$ in the same row if fol1owed by different letters. [*]Treatments are indicated as the amount of added sodium chloride and are shown in the top row.

Table 4: Carbohydrate concentration of dry seeds and germinated seedlings under increasing salt stress*. Total soluble carbohydrates were extracted from dry seed and salt stressed seedlings germinated in the presence of the indicated concentration of NaCl added to the medium. Carbohydrate concentration (μg/gfw) in the extracts was analyzed by HPLC. Data are means ± standard error for duplicate determinations. Statistical significance was tested using GLM.

Arabidopsis	+0mM	+50mM	+100mM	+150mM	+200mM	+300mM	Seed
Fructose	6 ± 0.19 a[z]	23 ± 0.19 b	0.0	0.0	0.0	0.0	22 ± 0.19 c
Glucose	91 ± 0.98 a	84 ± 0.98 a	35 ± 0.98 b	26 ± 0.98 b	22 ± 0.98 b	29 ± 0.98 b	220 ± 0.98 c
Sucrose	161 ± 38.0 a	247 ± 38.0 a	169 ± 38.0 a	498 ± 38.0 a	613 ± 38.0 a	512 ± 38.0 a	9726 ± 38.0 b
Mannitol	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Myo-inositol	34 ± 0.57 a	77 ± 0.57 b	30 ± 0.57 c	98 ± 0.57 d	109 ± 0.57 e	69 ± 0.57 f	159 ± 0.57 g
Raffinose	0.0	0.0	0.0	0.0	0.0	31 ± 2.83 a	986 ± 2.83 b
Stachyose	0.0	0.0	0.0	0.0	0.0	0.0	4253 ± 14.4 a
Total Carbohydrate	292	431	234	856	744	641	15366
Celery 1							
Fructose	581 ± 17.7 a	1376 ± 17.7 b	1075 ± 17.7 c	799 ± 17.7 d	911 ± 17.7 d	1222 ± 17.7 e	1834 ± 17.7 f
Glucose	357 ± 7.46 a	961 ± 7.46 b	748 ± 7.46 c	487 ± 7.46 d	538 ± 7.46 d	667 ± 7.46 e	608 ± 7.46 e
Sucrose	681 ± 58.9 a	1208 ± 58.9 a	3673 ± 58.9 b	8217 ± 58.9 c	8378 ± 58.9 c	6224 ± 58.9 d	6727 ± 58.9 e
Mannitol	220 ± 4.68 a	1019 ± 4.68 b	701 ± 4.68 c	168 ± 4.68 d	73 ± 4.68 e	93 ± 4.68 e	763 ± 4.68 f
Myo-inositol	327 ± 3.50 a	492 ± 3.50 b	395 ± 3.50 c	242 ± 3.50 d	211 ± 3.50 d	151 ± 3.50 e	470 ± 3.50 f
Raffinose	16 ± 1.75 a	22 ± 1.75 a	43 ± 1.75 b	70 ± 1.75 c	69 ± 1.75 c	37 ± 1.75 d	57 ± 1.75 e
Stachyose	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Carbohydrate	2182	5078	6635	9983	10180	8394	10459
Cabbage							
Fructose	351 ± 25.2 a	334 ± 25.2 a	187 ± 25.2 a	399 ± 25.2 a	510 ± 25.2 a	1011 ± 25.2 b	445 ± 25.2 c
Glucose	566 ± 9.65 a	566 ± 9.65 a	298 ± 9.65 b	646 ± 9.65 c	785 ± 9.65 d	1230 ± 9.65 e	576 ± 9.65 f
Sucrose	249 ± 10.4 a	315 ± 10.4 a	297 ± 10.4 a	920 ± 10.4 b	1318 ± 10.4 c	3793 ± 10.4 d	8672 ± 10.4 e
Mannitol	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Myo-inositol	130 ± 2.95 a	166 ± 2.95 b	131 ± 2.95 c	305 ± 2.95 d	392 ± 2.95 e	458 ± 2.95 f	288 ± 2.95 g
Raffinose	0.0	0.0	0.0	0.0	24 ± 3.35 a	182 ± 3.35 b	1519 ± 3.35 c
Stachyose	0.0	0.0	0.0	0.0	0.0	455 ± 9.38 a	6353 ± 9.38 b
Total Carbohydrate	1296	1381	913	2270	3029	7129	17853
Celery 2							
Fructose	350 ± 20.6 a	1167 ± 20.6 b	1049 ± 20.6 b	1189 ± 20.6 b	1919 ± 20.6 c	1412 ± 20.6 d	1726 ± 20.6 e
Glucose	249 ± 8.33 a	766 ± 8.33 b	619 ± 8.33 c	649 ± 8.33 c	1096 ± 8.33 d	728 ± 8.33 e	559 ± 8.33 f
Sucrose	641 ± 66.8 a	1917 ± 66.8 b	6178 ± 66.8 c	9589 ± 66.8 d	6770 ± 66.8 e	4915 ± 66.8 e	6013 ± 66.8 f
Mannitol	146 ± 3.97 a	896 ± 3.97 b	112 ± 3.97 c	84 ± 3.97 c	86 ± 3.97 c	62 ± 3.97 c	647 ± 3.97 d
Myo-inositol	211 ± 2.17 a	444 ± 2.17 a	221 ± 2.17 b	217 ± 2.17 c	188 ± 2.17 d	112 ± 2.17 d	120 ± 2.17 e
Raffinose	0.0	0.0	0.0	0.0	0.0	0.0	86 ± 5.48 a
Stachyose	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Carbohydrate	1597	5190	8179	11728	10059	7229	9151
Tobacco							
Fructose	60 ± 19.5 a	22 ± 19.5 a	388 ± 19.5 b	467 ± 19.5 b	102 ± 19.5 c	23 ± 19.5 c	0.0
Glucose	99 ± 48.2 a	90 ± 48.2 a	334 ± 48.2 a	450 ± 48.2 a	117 ± 48.2 a	48 ± 48.2 a	156 ± 48.2 a
Sucrose	284 ± 52.7 a	487 ± 52.7 a	1270 ± 52.7 b	4669 ± 52.7 c	2663 ± 52.7 d	3492 ± 52.7 e	5155 ± 52.7 f
Mannitol	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Myo-inositol	84 ± 6.68 a	180 ± 6.68 b	307 ± 6.68 b	405 ± 6.68 b	195 ± 6.68 c	83 ± 6.68 d	21 ± 6.68 d
Raffinose	0.0	0.0	0.0	0.0	0.0	0.0	61 ± 2.27 a
Stachyose	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total Carbohydrate	527	779	2299	5991	3077	3646	5393

[z]In the same row, means followed by different letters (e.g. a, b, c) are significantly different at $p < 0.0001$. *Sodium chloride added in each treatment is shown.

Figure 1: Seed germination under salt stress. Seeds of the salt tolerant (Celery 1, Celery 2, and Cabbage) and sensitive (Arabidopsis and Tobacco) taxa were germinated on media containing the indicated amounts of added NaCl. Numbers of germinated seeds were recorded daily for each repetition, treatment and taxa. Statistical regression curves were fit using Weibull analysis as described in Materials and Methods. Means are plotted as percent germination by day with the Weibull generated curve superimposed. Y-error bars represent standard deviation. Treatments were plotted as follows: (—■—) +0 mM; (—□—) +50mM; (¨●¨) + l00mM; (—○—) + l50mM. Where curves are absent regression failed.

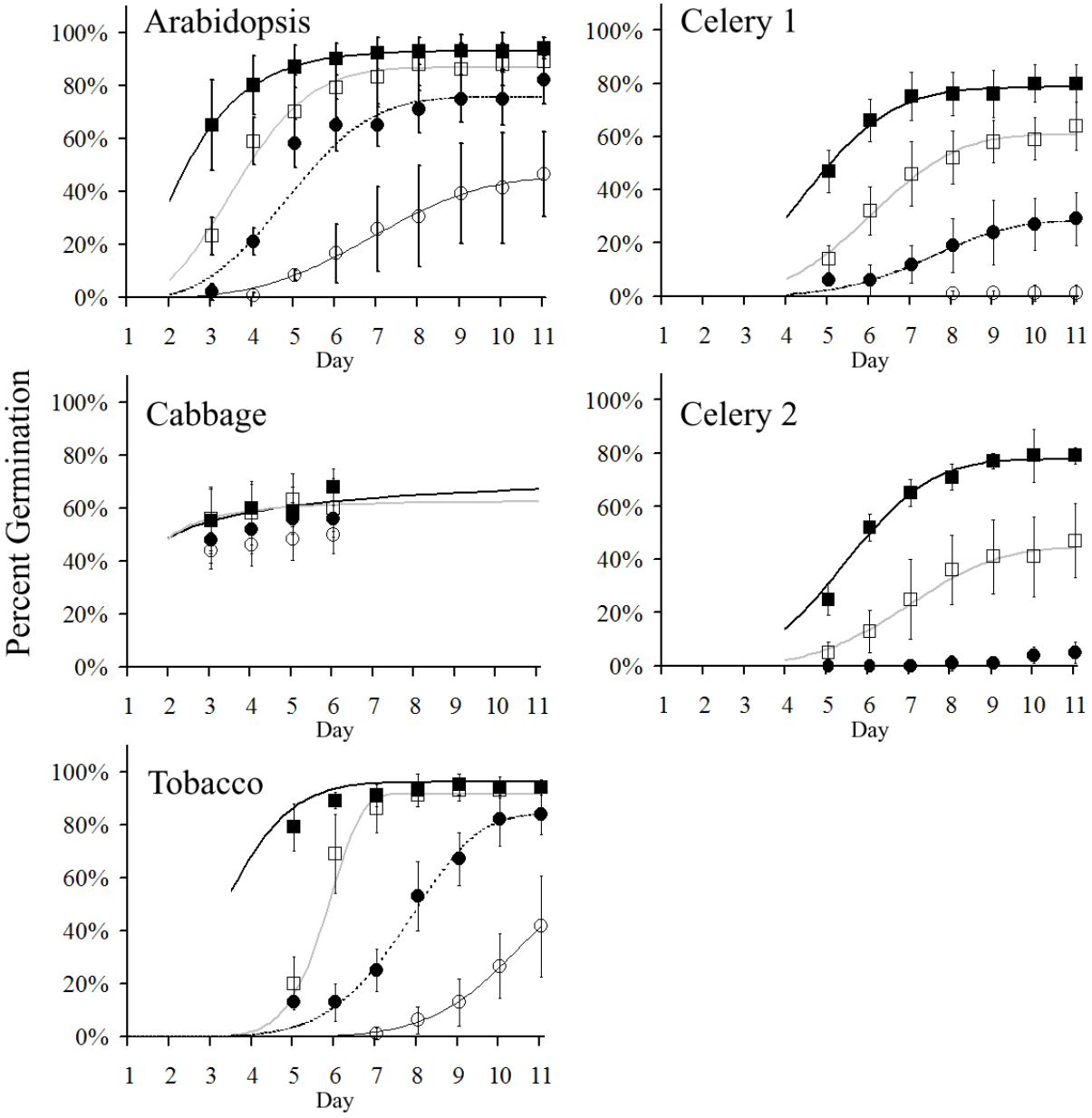
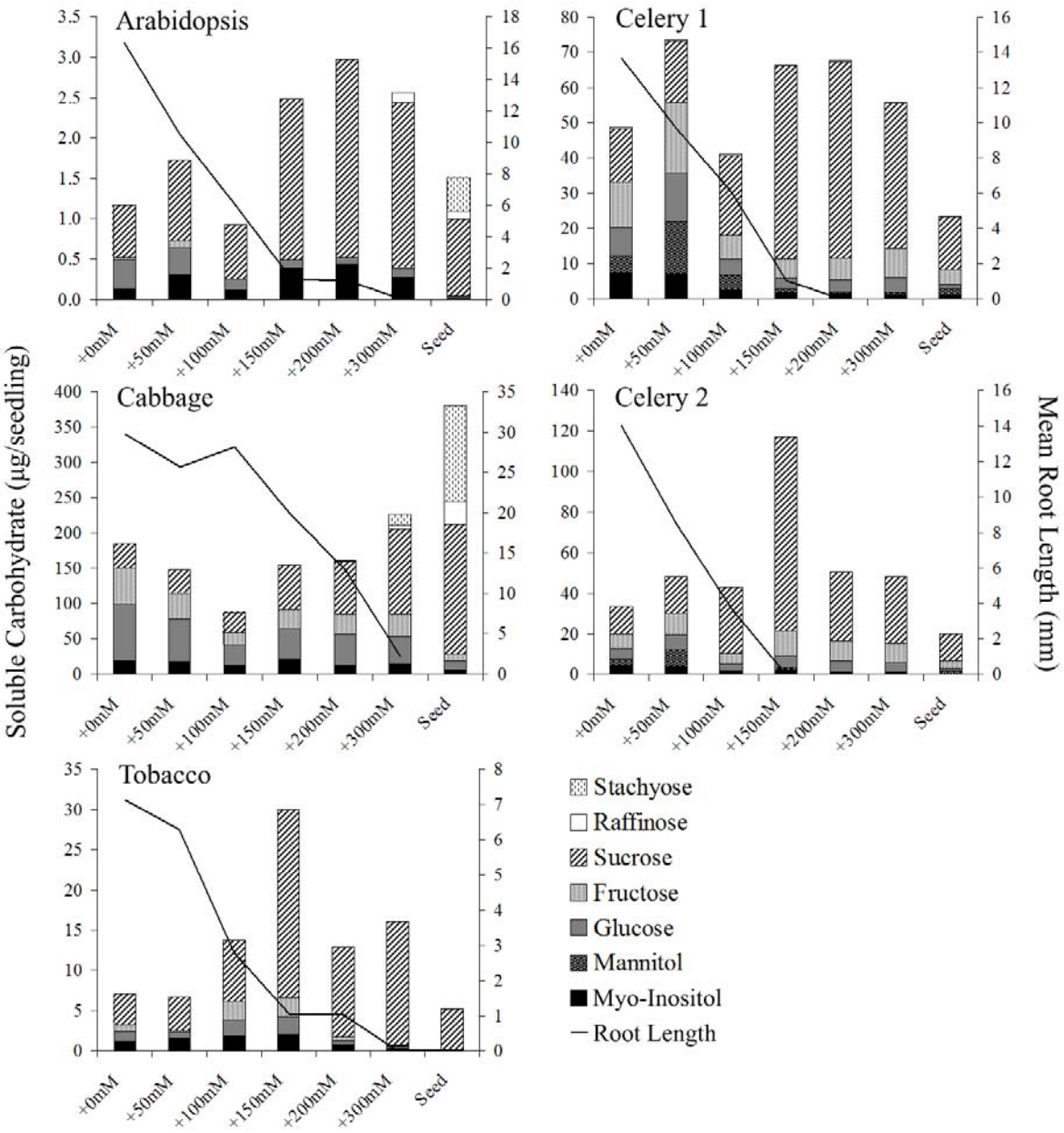


Figure 2. Carbohydrate content of dry seeds and seedlings under increasing salt stress. Seedlings of salt sensitive (Arabidopsis and Tobacco) and tolerant (Celery and Cabbage) taxa were grown under increasing levels of salt stress. Total soluble carbohydrates were extracted from dry seed and salt stressed seedlings and analyzed using HPLC. Final seedling root lengths were measured for each treatment and taxa combination. Carbohydrate amounts per seedling were calculated from carbohydrate concentrations in the HPLC samples. Means of root lengths for all blocks are plotted below (mm) on the secondary Y-axis (Solid line) Carbohydrate amounts and type are plotted on the primary Y-axis. Treatments are plotted on the X-axis.



Appendix

Figure 3: Final mean root lengths with corresponding photographs for germinating seeds of the non-mannitol plants Arabidopsis and tobacco. Mean root lengths (mm) for all blocks are plotted against increasing salt concentrations. Corresponding representative photographs of each treatment level from day 7 of growth are shown.

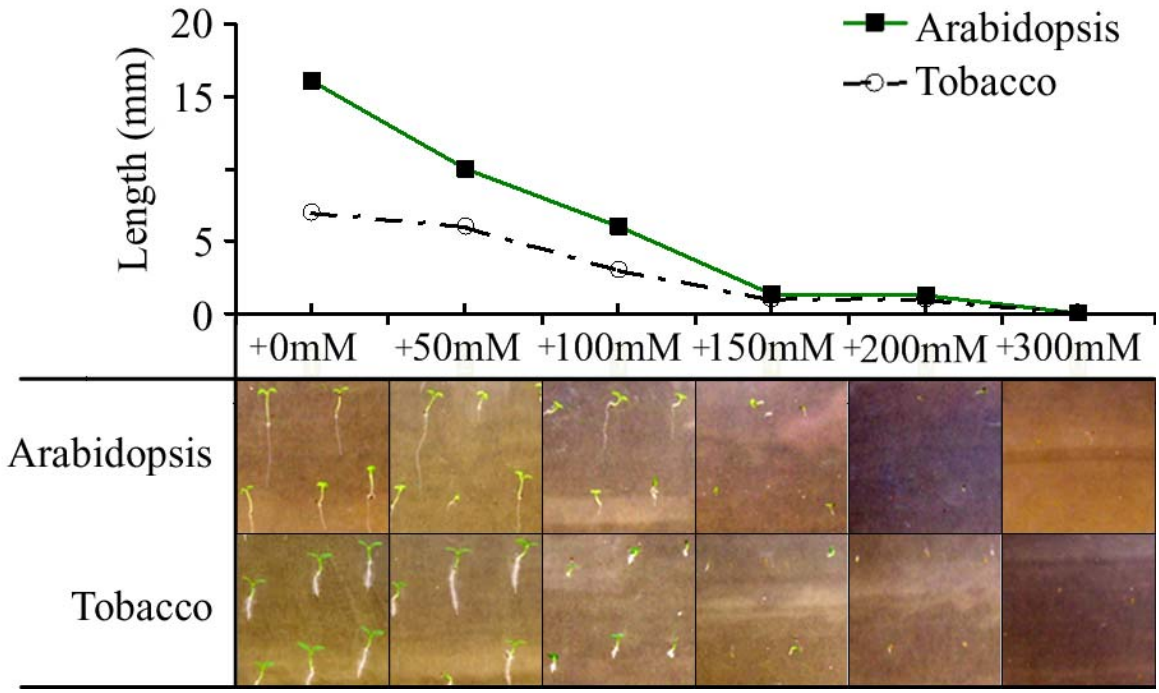


Figure 4: Final mean root lengths with corresponding photographs for seeds of the salt tolerant, non-mannitol plant cabbage Mean root lengths (mm) for all blocks are plotted against increasing salt concentrations. Corresponding representative photographs of each treatment level from day 4 of growth are shown.

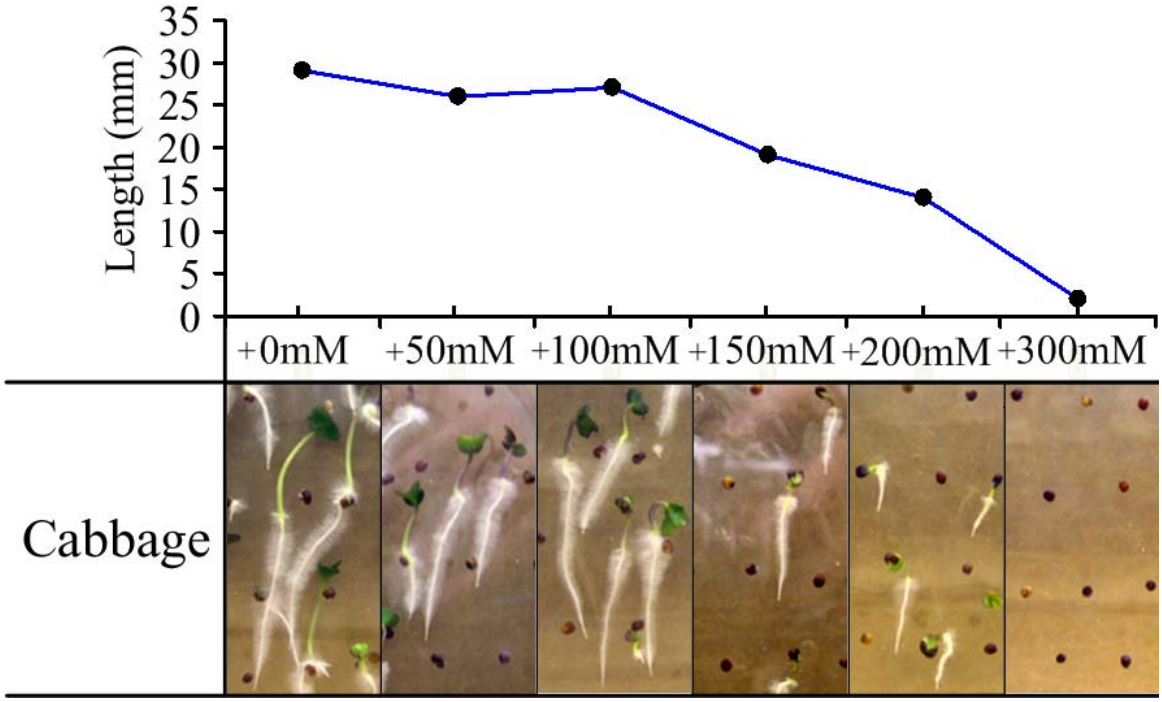


Figure 5: Final mean root lengths with corresponding photographs for seeds of the mannitol-containing salt tolerant plants, celery1 and 2. Plotted below are mean root lengths (mm) for all blocks plotted against increasing salt concentrations. Corresponding representative photographs of each treatment level from day 7 of growth are below.