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Characterization of Physiological Responses of Suspension Cells From Alligator Weed (Alternanthera Philoxeroides (Mart.); Griseb) to an Abrupt Increase in Salinity.

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*Louisiana State University and Agricultural & Mechanical College*

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Characterization of physiological responses of suspension cells from alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb) to an abrupt increase in salinity

Balagtas-Burow, Gloria, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1993
CHARACTERIZATION OF PHYSIOLOGICAL RESPONSES OF SOSPENSION CELLS FROM ALLIGATOR WEED (Alternanthera philoxeroides [Mart.] Griseb) TO AN ABRUPT INCREASE IN SALINITY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Botany

by
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ABSTRACT

Dark-grown suspension cells of alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb) were established in Murashige and Skoog medium and used to characterize several cellular responses to an abrupt increase in salinity (0 to 200 mol m\(^{-3}\) NaCl). The fresh weight and cell density of salt-treated cells of alligator weed more than doubled by 7 d. Cell viability declined to 77% by 1 d, but recovered to 86% by 7 d after salt treatment. These responses indicate that alligator weed cells survived and adapted to an abrupt increase in salinity. Osmotic potential (\(\psi_s\)) of salt-treated cells declined to the \(\psi_s\) of the salt treatment medium and symplastic volume decreased by 36% at 2 h. This decrease in \(\psi_s\) at 2 h was primarily due to increased \([Na^+]\) and \([Cl^-]\), although concentrations of \(K^+\), reducing sugars and quaternary ammonium compounds (QAC) also increased. From 2 h to 6 h after transfer to salt treatment, there was further but slower decline in cellular \(\psi_s\), an increase in symplastic volume and cellular turgor potential (\(\psi_p\)) became positive. By 3 d, \(\psi_p\) was similar in control and salt-treated cells and the fresh weight of salt-treated cells was increasing. Cellular concentrations of sucrose, proline and total free amino acids also increased in salt-treated cells at 7 d. Decreases in cellular \([Na^+]\) and \([Cl^-]\) between 1 and 7 d indicate efficient regulation of these ions in alligator weed cells during prolonged salinity. These responses to an abrupt increase in salinity were accompanied by increased staining and incorporation of \(^{35}\)S-methionine into two soluble
polypeptides and induction of two membrane polypeptides as early as 12 h after the beginning of salt treatment. The response of alligator weed cells was distinct from that of tobacco cells, which did not attain positive $\psi_p$ during the first 24 h, exhibit recovery of cell viability by 7 d or show an increase in fresh weight over the 21 d period after an abrupt increase in salinity. These results characterize several facets of cellular response to salinity and demonstrate the value of the alligator weed suspension system for studies of plant response to salinity.
CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

This dissertation presents a systematic characterization of physiological responses by cultured suspension cells from alligator weed, *Alternanthera philoxeroides* [Mart.] Griseb, to an abrupt increase in salinity. The five chapters include an introduction and review of the literature, three chapters on experimental studies and a conclusion chapter.

This chapter provides background information on the physiological responses of whole plants and on osmotic adjustment to salinity stress. A brief review of the physiological and biochemical studies that have utilized cultured cells to understand the cellular basis of salinity stress follows. Finally, the biology of alligator weed is reviewed and the objectives of the experimental studies reported in the dissertation are discussed.

GENERAL INFORMATION ON SALINITY STRESS

Salinity stress is a condition that refers to the occurrence of high concentrations of total dissolved salts in the rhizosphere of the plant, defined as concentrations high enough to lower the water potential (Ψ) by -0.05 to -0.1 MPa (Levitt, 1980). Salinity stress is produced by excessive amounts of various ions such as Na⁺, Cl⁻, Ca²⁺, Mg²⁺, and SO₄²⁻ (Taiz and Lincoln, 1991). Salinity stress has been differentiated from stress produced by high [Na⁺] only, technically...
referred to as sodicity stress (Levitt, 1980). However, the problems of salinity stress are commonly due to high levels of Na\(^+\) and Cl\(^-\) ions. In most studies, salinity stress refers to high NaCl unless otherwise specified. In this dissertation, salinity stress is used as a synonym for NaCl stress.

A large increase in NaCl concentration ([NaCl]) in the root zone adversely affects plant growth and productivity and is an important and widespread environmental problem. Specifically, salinity stress causes a 25 to 50% reduction in total yield in salt-sensitive crop species (Epstein, Norlyn, Rush, Kingsbury, Kelley, Cunningham and Wrona, 1980). It has been estimated that about one third of all cultivated land in the world is affected by high [Na\(^+\)] and [Cl\(^-\)] (Tanji, 1990). Most species which are considered agriculturally important are sensitive to high salt or increases in [NaCl] (examples are shown in Table 1.1). Historically, salinity stress was also important as one of the major factors in determining areas that were first inhabited and developed for agricultural cultivation (Hale and Orcutt, 1987). Because of these significant economic effects, understanding of the physiological responses and mechanisms of adaptation to salinity stress by higher plants are important.

Plants can be classified as halophytes or glycophytes (or non-halophytes), based on the level of salinity stress in their habitats and growth rates in medium supplemented with specific [NaCl] (Flowers, Troke and Yeo, 1977; Greenway and Munns, 1980). Glycophytes or non-halophytes are species that grow in soils with
Table 1.1. *Examples of glycophytes*

Examples of plant species considered to be glycophytes, the family to which they belong and the threshold [NaCl] that each species tolerates. The data for this table are from Tanji (1989).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Species</th>
<th>Family</th>
<th>Threshold [NaCl]* (mol m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean</td>
<td><em>Phaseolus vulgaris</em></td>
<td>Fabaceae</td>
<td>10</td>
</tr>
<tr>
<td>Corn</td>
<td><em>Zea mays</em></td>
<td>Poaceae</td>
<td>17</td>
</tr>
<tr>
<td>Peanut</td>
<td><em>Arachis hypogaea</em></td>
<td>Fabaceae</td>
<td>32</td>
</tr>
<tr>
<td>Rice</td>
<td><em>Oryza sativa</em></td>
<td>Poaceae</td>
<td>30</td>
</tr>
<tr>
<td>Soybean</td>
<td><em>Glycine max</em></td>
<td>Fabaceae</td>
<td>50</td>
</tr>
<tr>
<td>Sugarbeet</td>
<td><em>Beta vulgaris</em></td>
<td>Chenopodiaceae</td>
<td>70</td>
</tr>
<tr>
<td>Sugarcane</td>
<td><em>Saccharum officinarum</em></td>
<td>Poaceae</td>
<td>17</td>
</tr>
<tr>
<td>Sunflower</td>
<td><em>Helianthus annuus</em></td>
<td>Asteraceae</td>
<td>61</td>
</tr>
<tr>
<td>Wheat</td>
<td><em>Triticum aestivum</em></td>
<td>Poaceae</td>
<td>86</td>
</tr>
<tr>
<td>Broccoli</td>
<td><em>Brassica oleracea</em> var. botrytis</td>
<td>Brassicaceae</td>
<td>28</td>
</tr>
<tr>
<td>Carrot</td>
<td><em>Daucus carota</em></td>
<td>Apiaceae</td>
<td>10</td>
</tr>
<tr>
<td>Celery</td>
<td><em>Apium graveolens</em></td>
<td>Apiaceae</td>
<td>18</td>
</tr>
<tr>
<td>Onion</td>
<td><em>Allium cepa</em></td>
<td>Alliaceae</td>
<td>12</td>
</tr>
<tr>
<td>Potato</td>
<td><em>Solanum tuberosum</em></td>
<td>Solanaceae</td>
<td>17</td>
</tr>
<tr>
<td>Cucumber</td>
<td><em>Cucumis sativus</em></td>
<td>Cucurbitaceae</td>
<td>25</td>
</tr>
<tr>
<td>Tomato</td>
<td><em>Lycopersicon esculentum</em></td>
<td>Cucurbitaceae</td>
<td>25</td>
</tr>
</tbody>
</table>

*Threshold [NaCl] refers to the maximum [NaCl] that can be applied to the soil where the species is grown without causing 50% reduction in biomass yield.*
low levels of salt concentration usually containing 10 to 75 mol m$^3$ NaCl (Greenway and Munns, 1980). Glycophytes exhibit 30-80% reduction in growth and biomass yield in medium with 100 to 200 mol m$^3$ NaCl (Greenway and Munns, 1980). Glycophytes are further subdivided into salt-tolerant and salt-sensitive classes. Salt-tolerant and salt-sensitive classes respectively refer to species that exhibit 30% and 50% or more reduction in biomass at 100 mol m$^3$ NaCl (Greenway and Munns, 1980). Most higher plants are considered glycophytes, especially agriculturally important species (Table 1.1).

In contrast, halophytes are species native to saline soils (soil with water solution containing at least 200 mol m$^3$ NaCl) and complete their life cycles in this environment (Jennings, 1976). They exhibit greater than 100% growth rates in hydroponic medium with [NaCl] of 100 to 200 mol m$^3$ relative to growth rates in medium without NaCl (Greenway and Munns, 1980). A limited number of species that are considered halophytes. Some examples of halophytes, which had higher than 100% growth rate in 200 mol m$^3$ NaCl and the families to which they belong are shown in Table 1.2 (Flowers, Troke and Yeo, 1977; Flowers, 1985; Long and Baker, 1986).

**PHYSIOLOGICAL RESPONSES OF WHOLE PLANTS TO SALINITY STRESS**

Plant responses to high salinity are directed towards the three basic adverse effects of salinity stress. The basic problems due to salinity stress include: (a) water or osmotic stress due to lower osmotic potential ($\psi_o$) of the surrounding
Table 1.2.  *Examples of halophytes*
Examples of species classified as halophytes and the family to which they belong (Flowers, Troke and Yeo, 1977; Flowers, 1985 and Long and Baker, 1986). These species have been reported to have maximum growth rates at salinity concentrations greater than 100 mol m$^{-3}$ NaCl.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Species</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saltbush</td>
<td><em>Atriplex nummularia</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Sea Orach</td>
<td><em>Atriplex halimus</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Orach</td>
<td><em>Atriplex polycarpa</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Seabeach Orach</td>
<td><em>Atriplex arenaria</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Suaeda</td>
<td><em>Suaeda maritima</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Glasswort</td>
<td><em>Salicornia rubra</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Samphire</td>
<td><em>Salicornia europea</em></td>
<td>Chenopodiaceae</td>
</tr>
<tr>
<td>Seashore mallow</td>
<td><em>Kosteletzkya virginica</em></td>
<td>Malvaceae</td>
</tr>
<tr>
<td>Spike grass</td>
<td><em>Distichlis spicata</em></td>
<td>Poaceae</td>
</tr>
<tr>
<td>Marsh cord grass</td>
<td><em>Spartina alterniflora</em></td>
<td>Poaceae</td>
</tr>
<tr>
<td>Slough grass</td>
<td><em>Spartina pectinata</em></td>
<td>Poaceae</td>
</tr>
<tr>
<td>Beach aster</td>
<td><em>Aster trifolium</em></td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Ice plant</td>
<td><em>Mesembryanthemum crystallinum</em></td>
<td>Aizoaceae</td>
</tr>
<tr>
<td>Sea purslane</td>
<td><em>Sesuvium portulacastrum</em></td>
<td>Aizoaceae</td>
</tr>
<tr>
<td>Beach amaranth</td>
<td><em>Amaranthus pumilus</em></td>
<td>Amaranthaceae</td>
</tr>
</tbody>
</table>
environment, (b) specific ion toxicity and (c) ion imbalance or induced nutrient deficiencies. Salinity stress also affects important metabolic processes such as photosynthesis, respiration, and protein synthesis in various ways which are related to these three basic problems (Poljakoff-Mayber, 1982).

A primary effect of salinity stress is on plant water relations (Kramer, 1983). High [NaCl] can reduce $\Psi$ in the environment to below $\Psi$ in plants. This results in the movement of water from the plant to the environment causing dehydration and loss of turgor. To maintain water uptake and prevent dehydration, plants must be capable of lowering their $\psi_s$ and thus $\Psi$, as the rhizosphere $\Psi$ decreases (Boyer, 1968). The decline in level of $\psi_s$ of cells must be to values below the $\Psi$ of the environment so that the plants can maintain a positive turgor potential ($\psi_p$). Positive $\psi_p$ is critical to various plants processes especially cell enlargement (Boyer, 1970; Hsiao, Acevedo, Ferreres and Henderson, 1976; Acevedo, Ferreres, Hsiao and Henderson, 1979). Inhibitory effects of salinity stress on growth and biomass accumulation are directly related to these osmotic stress problems (Greenway and Munns, 1983; Kramer, 1983).

One of the most important physiological and adaptive responses to salinity stress is osmotic adjustment. Osmotic adjustment refers to a net increase in solute concentration independent of the change in volume of the cells (Hsiao et al., 1976; Munns, 1988). A plant is considered to adjust osmotically to salinity if $\psi_s$ of the cellular sap decreases by magnitude greater than the osmotic potential of the
outside solution thereby facilitating maintenance of positive turgor and preventing cellular dehydration. In higher plants osmotic adjustment is the result of uptake of ions from the environment and internal synthesis of organic solutes (Hellebust, 1976; Flowers et al., 1977; Wyn Jones and Gorham, 1983). An important hypothesis that is associated with osmotic adjustment involves intracellular compartmentation of solutes (Flowers et al., 1977; Dracup and Greenway, 1980).

High ion concentrations which are thought to be inhibitory to most metabolic pathways can be tolerated by cells because the Na⁺ and Cl⁻ ions are localized in the vacuole, and more neutral organic solutes contribute to the osmotic potential of the cytoplasm (Wyn Jones and Gorham, 1983; Hajibagheri, Harvey and Flowers, 1987). These neutral organic solutes are sometimes referred to as compatible solutes because they are less inhibitory to metabolic reactions than isosmotic concentrations of inorganic ions and are accumulated in high quantities at low external Ψ in several species (Wyn Jones and Gorham, 1983). Compatible solutes include sugars, sugar alcohols, amino acids like proline, quaternary ammonium compounds (eg., betaines, choline) and sulphonium compounds (Wyn Jones and Gorham, 1983). Accumulation of compatible solutes is specific, that is, certain species produce only a particular compatible solute (Flowers et al., 1977, Wyn Jones and Gorham, 1983). Furthermore only a limited number of plant species have the physiological capability to synthesize compatible solutes (Flowers et al., 1977). The significance and advantageous effects of osmotic adjustment are well
established, but the mechanisms involved in the process are not well understood (Munns, 1988).

Descriptions of physiological responses of whole plants to salinity are extensive and complex (Flowers et al., 1977; Dracup and Greenway, 1980). However, because of the complexity and the experimental differences at the whole plant level, an understanding of the physiological responses and mechanisms of adaptation to salinity stress is not complete. For example, ion accumulation during salt treatment involves uptake by roots, transport into the vascular tissues and accumulation in the leaves, which are processes that involve different types of cells and transport mechanisms. The overall effect of salinity could be attributed to any of the processes and physiological responses involved in each step (Dracup and Greenway, 1980; Ben-Hayyim, Kafkafi and Ganmore-Neumann, 1987). Because of these intrinsic problems with whole plants, the use of simpler systems like cultured cells to study physiological responses to salinity stress have become increasingly frequent.

STUDIES ON SALINITY STRESS USING CELL CULTURES

Cell cultures are advantageous for studies of physiological responses to salinity stress because they are simpler systems that minimize the complications and problems encountered with whole plants (Stavarek and Rains, 1984; Lerner, 1985, Hasegawa, Bressan and Handa, 1986). Several important features of cell cultures that make them very useful for studies of physiological responses and
adaptation to salinity stress have been recognized (Stavarek and Rains, 1984; Dracup, 1991). First, cell cultures provide cells with fairly uniform genotype and developmental stage. Second, cell cultures facilitate greater precision in controlling the nutritional and osmotic environment experienced by these cells. Third, large number of cell culture samples can be analyzed using less space and shorter time periods.

Cell cultures have been used in attempts to improve salt tolerance in agriculturally important glycophytic species. Salt tolerant callus cultures have been selected from various glycophytic species such as *Nicotiana sylvestris* and *Capsicum annum* (Dix and Street, 1975), *Nicotiana tabacum* cultivar Wisconsin 38 (Hasegawa, Bressan and Handa, 1980; Watad, Reinhold and Lerner, 1983) and Var. Samsun (Nabors, Gibbs, Bernstein and Meis, 1980), *Oryza sativa* (Flowers et al., 1985), *Pennisetum americanum* (Rangan and Vasil, 1983), *Citrus sinensis* (Ben-Hayyim and Kochba, 1983), *Cicer arietinum* L. (Pandey and Ganapathy, 1984), *Pennisetum purpureum* (Chandler and Vasil, 1984), *Medicago sativa* (Croughan, Stavarek and Rains, 1978), *Vigna radiata* (Kumar and Sharma, 1989) and *Solanum tuberosum* (Sabbah and Tal, 1990). One significant problem with this approach is that regeneration of actual salt-tolerant plants from cell cultures is difficult. In addition, plants regenerated from salt-tolerant cultures do not necessarily exhibit tolerance to high salinity (McCoy, 1987; Dracup, 1991). Such results indicate that a better understanding of physiological responses and
mechanisms of tolerance to high salinity in cell cultures must first be established (Dracup, 1991).

Studies on physiological responses to high salinity have utilized cell cultures from glycophytic and halophytic species. High salinity treatment ranging from 100 to 340 mol m\(^{-3}\) NaCl reduced growth in a number of cell cultures, both callus and liquid cell suspension systems (Von Hedenstrom and Breckle, 1974; Hasegawa \textit{et al.}, 1980; Heyser and Nabors, 1981; Warren and Gould, 1982; Ben-hayyim and Kochba, 1983; Binzel, Hasegawa, Handa and Bressan, 1985; Sabbah and Tal, 1990; Casas, Bressan and Hasegawa, 1991; Blits, Cook and Gallagher, 1993). Based on these studies, the biomass of cell cultures from glycophytes are lower in salt treatment than in basal medium without NaCl (Hasegawa \textit{et al.}, 1980; Heyser and Nabors, 1981; Ben-Hayyim and Kochba, 1983; Binzel \textit{et al.}, 1985; Sabbah and Tal, 1990). Usually, fresh weight is more significantly affected than dry weight (Binzel \textit{et al.}, 1985). Reduction in cell fresh weight due to salinity could be a consequence of reduced cell volume due to limited cell expansion as is the case for tobacco, a glycophyte (Binzel \textit{et al.}, 1985), or reduced rate of cell division as is the case for potato (Sabbah and Tal, 1990), or possibly a combination of these factors. More importantly, these studies show that cell cultures from many glycophytic species do not tolerate large increases in [NaCl] (Hasegawa \textit{et al.}, 1980; Heyser and Nabors, 1981; Ben-Hayyim and Kochba, 1983; Sabbah and Tal, 1990). As an example, salt-adapted cell suspensions of
tobacco were produced after exposure to gradually increasing salinity at increments of 25 mol m$^{-3}$ NaCl per cultural generation (Hasegawa et al., 1980; Binzel et al., 1985). However, suspension cells from halophytes were shown to tolerate increases of 170 or 200 mol m$^{-3}$ NaCl (Warren and Gould, 1982; Warren et al., 1985; Blits et al., 1993).

Changes in water relations parameters are fundamental responses to salinity at the cellular level. The $\psi_*$ and $\psi_p$ of salt-adapted tobacco cells have been analyzed using incipient plasmolysis methods, osmometer measurements and turgor probe (Binzel et al., 1985; Dracup and Greenway, 1988). Tobacco cultures gradually adapted to high [NaCl] (by stepwise increases of 25 mol m$^{-3}$ NaCl) exhibited decreases in $\psi_*$ along with maintenance of positive $\psi_p$ (Binzel et al., 1985). Attainment of low cellular $\psi_*$ (compared to the medium $\psi_*$) was important towards avoiding cellular dehydration, while maintenance of $\psi_p$ was important for cell enlargement (Binzel et al., 1985; Dracup and Greenway, 1988).

Osmotic adjustment and accumulation of solutes has been investigated in cell cultures from various species, including tobacco (Binzel, Hasegawa, Rhodes, Handa, Handa and Bressan, 1987; Dracup and Greenway, 1988), carrots and sugar beet (Reuveni, Lerner and Poljakoff-Mayber, 1991), Distichlis spicata (Daines and Gould, 1985), Mesembryanthemum crystallinum (Thomas, DeArmond and Bohnert, 1992) and Kosteletzkya virginica (Blits et al., 1993). The major solutes accumulated by these cells include Na$^+$ and Cl$^-$ ions which account for more than
50% of the total cellular $\psi_s$. The significance of organic solutes, specifically proline, has also been shown in *D. spicata* (Daines and Gould, 1985) and *M. crystallinum* (Thomas, DeArmond and Bohnert, 1992). Other possible solutes that could be significant for osmotic adjustment of cells are organic acids, sugars and quaternary ammonium compounds like betaine. The compartmentation of solutes in cells has been analyzed in cell cultures of tobacco (Binzel, Hess, Bressan and Hasegawa, 1988) and sugar beet and carrot (Reuveni *et al.*, 1991). In salt-adapted tobacco cells, $\text{Na}^+$ and $\text{Cl}^-$ ions were highly localized in the vacuole, but were also present at concentrations of 80 to 100 mol m$^{-3}$ in the cytoplasm (Binzel *et al.*, 1988). Reuveni and coworkers (1991) observed that organic solutes, sucrose and proline accumulate in the cytoplasm of sugarbeet and carrot cells after 3 d of salt treatment. However, studies involving the time course of solute accumulation necessary for understanding the dynamics of osmotic adjustment in cell cultures exposed to high salinity have been generally lacking.

Studies on changes in protein synthesis using cell culture have also been carried out in order to determine the molecular basis of tolerance to salinity stress. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), an early study of tobacco suspensions found that two polypeptides with molecular weights ($M_r$) of 26,000 and 30,000 were more abundant in salt-adapted than unadapted cells (Ericson and Alfinito, 1984). In tobacco cells gradually adapted to 340 and 420 mol m$^{-3}$ NaCl, several polypeptides and a prominent 26 kD protein,
osmotin, were found to increase in salt adapted cells only (Singh, Handa, Hasegawa and Bressan, 1985; King, Hussey and Turner, 1986). Two polypeptides, 25 and 27 kD, increased in citrus and tomato cells adapted to 200 mol m$^{-3}$ NaCl (Ben-Hayyim, Vaadia and Williams, 1988). These two polypeptides identified from tomato and citrus did not cross react with antibody against tobacco osmotin (Ben-Hayyim, Vaadia and Williams, 1988). Fifteen polypeptide spots showed increased labeling in sugarcane cells adapted to 257 and 342 mol m$^{-3}$ NaCl compared to unadapted cells using two dimensional gel electrophoresis (Ramagopal and Carr, 1991). These studies suggest that the molecular basis of salinity stress is relatively complex and is associated with induction of a number of proteins. An important consideration from these results is that the actual metabolic roles of the polypeptides that are enhanced or only produced under salinity stress are not known. That is, while it is assumed that these polypeptides play a role in salt adaptation, the specific processes in which they might participate have yet to be discovered.

There is also limited information on physiological responses of suspension cells to an abrupt increase in salinity. Dracup (1991) suggested that studies dealing with physiological responses to a rapid and large increase in salinity be the basis for understanding the regulation of $\psi_p$ and the role of such regulation in tolerance to high salinity in suspension cells. Cell cultures of the halophytes *Suaeda maritima*, *D. spicata*, *S. pectinata* and *M. crystallinum* have been studied for their
growth responses to a one-step increase in [NaCl] from 0 to 170 or 200 mol m$^{-3}$
NaCl (von Hedenstrom and Breckle, 1974; Warren and Gould, 1982; Warren et al., 1985; Thomas et al., 1992). Results from these studies showed that cell cultures from these species readily tolerate such an abrupt increase in salinity by continuing to grow and increase in biomass over a 21 to 40 day period. However, these studies have focused mainly on growth analysis and have not systematically characterized the physiological responses related to osmotic adjustment per se.

PHYSIOLOGICAL RESPONSES OF ALLIGATOR WEED TO SALINITY STRESS

Alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb, is a dicotyledonous C3 member of the family Amaranthaceae. Alligator weed is found in diverse habitats in the southeastern United States (Coulson, 1977; Gangstad, 1978). Alligator weed forms mats of both erect and prostrate stems that float on running or still water. Although it appears to be an aquatic plant, the roots of alligator weed are anchored in shallow sediments or in the soil bordering the edge of waterways. Alligator weed also thrives in brackish marshes with moderate levels of salinities and in drier environments (Sculthorpe, 1967). It is considered a salt-tolerant species and was reported to survive under conditions of fluctuating levels of salinity (Coulson, 1977).

A notable characteristic of this species is that it adapts to a variety of environments and is easy to establish in different geographical areas (Gangstad,
An increase in average dry weight per unit leaf area with increasing light intensity as well as ability to grow and produce significant biomass even under low light intensity was demonstrated for alligator weed in the laboratory (Longstreth and Mason, 1984). Physical and morphological features of alligator weed leaves changed with exposure to different light levels, but at lower light intensity alligator weed compensates for some of the morphological changes and exhibits lower but substantial photosynthetic rates (Longstreth, Bolaños and Goddard, 1985). These observations further show that alligator weed adapts easily not only to different environments but to adverse conditions as well.

Alligator weed is easy to propagate vegetatively and is readily established in hydroponic culture. In hydroponic culture, alligator weed plants continued to grow at increasing salinities up to 400 mol m^{-3} NaCl with reduced photosynthetic rates (Longstreth, Bolaños and Smith 1984). Alligator weed plants adjust osmotically and maintain water movement from the rhizosphere to roots allowing growth at [NaCl] of 400 mol m^{-3} NaCl (Bolaños and Longstreth, 1984). High [NaCl] also increases the bulk elastic modulus of shoot tissue of alligator weed (Bolaños and Longstreth, 1984). Chloride and glycine betaine concentrations significantly increase in salt-treated leaves of alligator weed after 24 h of salt treatment (Su, 1986). These data indicate that alligator weed is capable of osmotic adjustment to large increases in salinity. Furthermore, alligator weed may be unique because it tolerates high salt treatment but is not normally found in saline habitats.
Adjustment to high salinity by alligator weed may involve mechanisms not found in glycophytes or true halophytes. Therefore, suspension cell cultures have been established to better understand the cellular mechanisms involved in the physiological responses of alligator weed to an abrupt increase in salinity.

The first objective of this study (Chapter 2) was to characterize growth and water relations of alligator weed suspension cells after exposure to an abrupt increase in salinity (0 to 200 mol m\(^{-3}\) NaCl). The growth response of alligator weed cells was then compared to tobacco, a well-studied glycophyte in order to determine whether the response of alligator weed to an abrupt increase in salinity can be differentiated from a well-characterized glycophyte suspension system. The salinity treatment used in this study was 200 mol m\(^{-3}\) NaCl so as to make the salinity treatments comparable to salinity levels used in studies at the whole plant level for other salt tolerant species.

To better understand osmotic adjustment in alligator weed cells, establishment of changes in solute concentrations are highly important. The goals of Chapter 3 were to quantify the major solutes and evaluate their contribution to osmotic adjustment in alligator weed cells after an abrupt increase in salinity. The objective of Chapter 4 was to analyze protein concentrations and polypeptide patterns during exposure to an abrupt increase in salinity. An examination of the changes in polypeptide pattern during adjustment to salinity could provide valuable information for understanding the regulation of osmotic adjustment. In Chapter 5,
a comprehensive description of the physiological responses of alligator weed to an increase from 0 to 200 mol m$^{-3}$ NaCl (Chapters 2 to 4) is presented.

LITERATURE CITED


LERNER HR. 1985. Adaptation to salinity at the plant cell level. Plant and Soil 89, 3-14.


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Louisiana State University  
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CHAPTER 2

GROWTH AND OSMOTIC ADJUSTMENT OF CULTURED SUSPENSION CELLS FROM *Alternanthera philoxeroides* [Mart.] Griseb AFTER AN ABRUPT INCREASE IN SALINITY

INTRODUCTION

Plant cells must adjust osmotically to maintain a positive water balance after an increase in salinity. The importance of osmotic adjustment is recognized but the mechanisms involved in this response are not well understood (Munns, 1988). The osmotic environment of cells in intact plants are generally heterogenous and therefore difficult to measure or manipulate (Handa, Bressan, Handa, Carpita and Hasegawa, 1983). One approach to minimizing these difficulties encountered in intact plants has been to study osmotic adjustment using suspension culture cells. Suspension cells are advantageous because the osmotic and nutritional environment is easily controlled and cell developmental age is relatively uniform (Binzel, Hasegawa, Handa and Bressan, 1985; Dracup and Greenway, 1988). While certain characteristics of cultured cells may vary from their counterparts in intact plants (McCoy, 1987a; McCoy, 1987b), the mechanisms of osmotic adjustment may be more readily dissected in suspension cell systems than in whole plant systems. The overall purpose of this study was to characterize the growth and water relations of a suspension cell system developed from a plant species capable of adjustment to significant increases in salinity.
Suspension systems have been used to study cellular adaptation to high salinity in a variety of plant species (e.g. von Hedenström and Breckle, 1974; Hasegawa, Bressan and Handa, 1980; Warren and Gould, 1982; Binzel et al., 1985; Daines and Gould, 1985; Warren, Baird and Thompson, 1985; Dracup and Greenway, 1988; Casas, Bressan and Hasegawa, 1991). When suspension cells from glycophytic species are subjected to an increase in media salinity of 100 mol m⁻³ NaCl or more, there is a significant reduction in biomass production and delay in the onset of growth (Hasegawa et al., 1980; Heyser and Nabors, 1981; Binzel et al., 1985). Salt-adapted suspension cultures can be produced from glycophytic species but only by gradual increases in medium NaCl concentration (increments of 25 mol m⁻³ per cultural generation are typical). Suspension cells from the halophytes, Suaeda maritima (von Hedenström and Breckle, 1974), Distichlis spicata (Warren and Gould, 1982) and Spartina pectinata (Warren et al., 1985), however, readily tolerate increases of 170 mol m⁻³ NaCl or 200 mol m⁻³ NaCl in media salinity. In general, however, there is limited information on osmotic adjustment of suspension cells to an abrupt increase in salinity. Understanding how suspension systems respond to an abrupt increase in salinity may provide insight into cellular adaptation to high salinity.

We have developed suspension cell cultures of alligator weed, Alternanthera philoxeroides [Mart.] Griseb, a dicotyledonous, C3, plant species. Intact plants have been shown to survive 400 mol m⁻³ NaCl (Longstreth, Bolaños and Smith,
This study focuses on the response of alligator weed suspension cells, not exposed previously to high salinity, to a step increase of 200 mol m$^3$ NaCl. Our specific objectives were to (1) characterize fresh weight and cell viability in salt-treated alligator weed as compared to tobacco (Nicotiana tabacum L.), a well studied suspension system, (2) evaluate the effect of salt treatment on weight, density, and volume of alligator weed cells and (3) determine water potential components of salt-treated alligator weed during the time course of osmotic adjustment.

MATERIALS AND METHODS

Cell Culture Conditions and Treatments

Cultured suspension cells derived from alligator weed leaf callus and tobacco callus (N. tabacum cv. Wisconsin 38 obtained from Carolina Biological Supply Company, Burlington, North Carolina) were grown in Murashige and Skoog (MS) liquid medium, containing 1 g m$^{-3}$ 2,4-D (Murashige and Skoog, 1962). For all experiments, 20 cm$^3$ of cell suspension, containing approximately 3.0 g fresh weight of cells, were transferred to a 250 cm$^3$ Erlenmeyer flask with 100 cm$^3$ of MS medium. Cultures were grown at 25°C in the dark on a rotary shaker set at 125 rpm. Seven-day old cultures, not previously exposed to NaCl, were subcultured to flasks containing either MS (control) or NaCl in MS to produce a final concentration of 200 mol m$^3$ (salt treatment). At each sampling time, 10 cm$^3$ of cell suspension
(per flask) were harvested from three flasks for each species growing in each
treatment. Experiments were repeated twice.

**Fresh and Dry Weight Measurements**

Fresh weight measurements were based on a method previously described
(Tanino, Weiser, Fuchigami and Chen, 1990) that minimizes error introduced by
media being trapped between cells when suspensions are packed (Dracup, 1991).
Briefly, 10 cm³ aliquots of suspension cells were transferred to graduated conical
tubes and centrifuged at 550g for 5 min at 25°C. Pelleted cells were transferred to
preweighed 5 cm³ plastic syringe barrels lined with two layers of absorbent paper.
The barrels were placed tip first into 15 cm³ conical centrifuge tubes. The tubes,
containing the syringes and cells, were then centrifuged at 800g for 5 min in a
swinging bucket rotor to expel extracellular water through the tips of the syringes
into the centrifuge tubes. We used 800g for this study because preliminary
measurements of fresh weight at 6 different speeds, ranging from 22g to 800g,
demonstrated that the fresh weight measurement became constant for both control
and salt-treated cells when centrifuged at speeds of 550g to 800g. Fresh weight was
taken as the final weight of the cell pellet after centrifugation and expressed per cm³
of suspension solution.

Dry weights were measured on cell pellets dried to constant weight in an
oven at 60°C. Ash weights were determined from dried samples heated in a muffle
furnace at 600°C for 12 h. Since cells were not washed, we calculated the effect of
differences in the concentration of treatment NaCl on ash-free weight. If the
concentration of NaCl in the apoplastic space equalled that in the treatment media,
then a maximum of approximately 5% of the difference between control and salt-
treated cells in ash-free weight was due to differences in the concentration of NaCl
in the cell apoplastic space.

Cell Density and Viability Assays

Cell density was determined by suspending 100 mg of pelleted cells (550g for
5 min) in 1.0 cm³ of 18% (w/v) chromic acid. This cell mixture was then incubated
at 60°C for 30 min, and then vigorously mixed with a Vortex mixer (Van Waters
and Rogers, Bohemia, New York) to disperse cell clumps. The average number of
cells in 10, 100 mm³ samples was determined using a hemacytometer (American
Optical, Buffalo, New York). Cell density was determined for three flasks for each
treatment at each time.

Cell viability was determined at two different times after subculture using
trypan blue stain (Kruse and Patterson, 1973). Briefly, 100 mg of pelleted cells
(550g for 5 min) were suspended in 1.0 cm³ of 0.4% (w/v) trypan blue (Sigma
Chemical Co., St. Louis, Missouri) and allowed to stand for 2 min. Cells were then
centrifuged, the supernatant discarded and the pellet resuspended in the appropriate
treatment medium. This process was repeated three times to wash out excess trypan
blue. Cell clumps were minimized with 2 passages of the solution through a 22
gauge stainless steel syringe needle at approximately 0.2 cm³ s⁻¹. Trypan blue is
excluded by intact membranes and therefore clear cells were counted as viable and blue cells as nonviable. For each sample, the average number of viable and nonviable cells was determined in 10, 100 mm³ samples using the hemacytometer.

Determination of Cell Symplastic Volume

Cell symplastic volume was determined using a modified silicon-oil centrifugation technique (Robinson, 1985). Cell suspensions were centrifuged at 550g, and 50 mg of cells were resuspended in appropriate media and labeled with either ³H-water or ³H-sorbitol. After 30 s of exposure to the label, cells were centrifuged through silicon oil and pelleted in 10% sucrose. The labeled cell pellets were resuspended and counted in scintillation cocktail (Liquiscint, National Diagnostics, Manville, New Jersey). The volume of cells labeled with ³H-water was taken as the total cell volume (V̅ₓTOT), while the volume of cells labeled with ³H-sorbitol was taken as the cell apoplastic volume (V̅ₐPO). Symplastic volume (V̅ₚₘ) was calculated as the difference between V̅ₓTOT and V̅ₐPO.

Water Potential Measurements

For estimation of the osmotic potential of the symplastic solution, suspension cell samples were centrifuged using the syringe-barrel technique to eliminate the significant volume of media normally trapped between cells. Cell pellets were transferred to capped, 1.5 cm³ centrifuge tubes, frozen in liquid N₂, placed in boiling water for 15 min and then centrifuged at 8000g for 1 min. Osmotically active solute concentrations of the supernatant and the growth medium were
determined from measurements with a freezing point osmometer (Microosmette, Precision Systems Inc., Nattick, Massachusetts) and the osmotic potential was calculated using the Van't Hoff relation (Nobel, 1991). The osmotic potential of the resulting supernatant ($\psi_x^{TOT}$) was the average osmotic potential of the cellular apoplastic ($\psi_x^{APO}$) and symplastic ($\psi_x^{SYM}$) solutions (Tomos, Leigh, Shaw and Wynn-Jones, 1984). We assumed that the cell water potential was in equilibrium with the medium and $\psi_x^{APO}$ equalled the medium osmotic potential. The cell osmotic potential was taken as $\psi_x^{SYM}$ and calculated from:

$$\psi_x^{TOT} \times V^{TOT} = (\psi_x^{SYM} \times V^{SYM}) + (\psi_x^{APO} \times V^{APO}).$$

Turgor potential was taken as the difference between water potential and cell osmotic potential.

RESULTS

Comparison of Alligator Weed and Tobacco Growth

The salt treatment (200 mol m$^{-3}$ NaCl in MS) reduced growth in both alligator weed and tobacco but the effect on tobacco was more inhibitory (Fig. 2.1). The doubling time for suspension fresh weight was approximately 4 d for both species in control (MS media) and the fresh weight at the end of the experiment was similar for both species. Fresh weight of the salt-treated alligator weed suspension doubled by 7 d but had not doubled in the salt-treated tobacco suspension by 21 d, the end of the experiment (Fig. 2.1). There was a threefold increase in the fresh weight of the salt-treated alligator weed suspension over the course of the experiment.
Figure 2.1. Fresh weight of alligator weed (A) and tobacco (B) after subculture to control (MS) or salt (200 mol m\(^{-3}\) NaCl in MS). Each point represents the mean of three replicates ± SD.
The mean fresh weight of both tobacco and alligator weed suspensions declined over the first 24 h after subculture to high salinity. The drop in tobacco fresh weight was 39%, which was much greater than the 6% drop in alligator weed fresh weight. The early difference in fresh weight between species was further analyzed with measurements of cell viability (Table 2.1). Cell viability was reduced to 77% in salt-treated alligator weed and to 42% in salt-treated tobacco after 1 d of subculture. After 7 d, the viability of salt-treated cells of alligator weed recovered to 86% while tobacco only recovered to 47%.

**Characteristics of Alligator Weed Cells**

Cell density was greater in the control treatment than in the salt treatment at all sampling times after subculture (Fig. 2.2). Cell density appeared to double by about 4 d in control suspensions and about 6 d in salt suspensions.

The fresh and dry weight of alligator weed cells was calculated from suspension weight and cell density measurements (Fig. 2.3). Fresh weight of salt-treated cells dropped below that of control cells by 24 h after subculture and the difference remained relatively constant throughout the remainder of the experiment (Fig. 2.3A). At 21 d, the fresh weight of salt-treated cells was 25% less than that of control cells. In contrast, the dry weight of salt-treated cells was greater than that of control cells by 7 d after subculture (Fig. 2.3B). The differences in dry weight between salt-treated and control cells remained relatively constant throughout the experiment with the dry weight of salt-treated cells being 14% greater than that
Table 2.1. *Viability of alligator weed and tobacco suspension cells*

Cells were grown in control or salt-treatment media at different times after subculture from MS. Values are means of three replicates (± SD).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Alligator weed</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>91.6 (1.15)</td>
</tr>
<tr>
<td>Salt</td>
<td>77.4 (1.95)</td>
</tr>
<tr>
<td><strong>Tobacco</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90.3 (1.42)</td>
</tr>
<tr>
<td>Salt</td>
<td>41.6 (0.81)</td>
</tr>
</tbody>
</table>
Figure 2.2. Cell density of alligator weed suspensions after subculture to control (MS) or salt (200 mol m$^{-3}$ NaCl in MS). Each point represents the mean of three replicates ± SD.
Figure 2.3. Fresh weight (A) and dry weight (B) of alligator weed cells after subculture to control (MS) or salt (200 mol m$^{-3}$ NaCl in MS). Each point represents the mean of three replicates ± SD.
of control cells at 21 d. Ash weights of salt-treated cells were 5-8% greater than that of control cells throughout the experiment (data not shown).

The symplastic volume of control cells remained relatively constant throughout the experiment (Fig. 2.4). In salt-treated cells, however, the symplastic volume declined over 36% within the first 2 h after subculture and then slowly increased (Fig. 2.4 inset). At 3 d after subculture and thereafter, the symplastic volume of salt-treated cells remained about 10% lower than that of control cells.

**Analysis of Alligator Weed Water Potential Components**

The media osmotic potential increased substantially during the experiment in both control and salt treatments, apparently due to nutrient uptake by cells (Fig. 2.5A). The osmotic potential of control cells was relatively constant averaging -0.63 MPa throughout the experiment. The osmotic potential of salt-treated cells declined to -1.50 MPa within one day, and remained relatively constant throughout the remainder of the experiment (Fig. 2.5A). The turgor potentials of control and salt-treated cells increased from day 1 through day 21 (Fig. 2.5B). Turgor potentials were similar in the two treatments after 1 d.

Since salt-treated cells appeared to have adjusted osmotically by 1 d in the first experiment (Fig. 2.5A), water relations parameters were determined at different times during the first 24 h after subculture in another experiment. The osmotic potential of salt-treated cells decreased to a value similar to that of the media by 2 h after subculture (Fig. 2.6A). Subsequently, the osmotic potential of
Figure 2.4. Symplastic volume of alligator weed cells at different times after subculture to control (MS) or salt (200 mol m$^{-3}$ NaCl in MS). Inset shows symplastic volume during the first 24 h. Each point represents the mean of three replicates ± SD.
Figure 2.5. Osmotic potential ($\psi_o$) of media (dotted lines) and cells (solid lines) (A) and turgor potential ($\psi_p$) (B) of alligator weed cells for a period of 21 d after subculture to control (MS) or salt (200 mol m$^{-3}$ NaCl in MS). Each point represents the mean of three replicates ± SD.
Figure 2.6. Osmotic potential ($\psi_\pi$) of media (dotted lines) and cells (solid lines) (A) and turgor potential ($\psi_p$) (B) of alligator weed cells for a period of 24 h after subculture to control (MS) or salt (200 mol m$^{-3}$ NaCl in MS). Each point represents the mean of three replicates ± SD.
continued to decrease slowly from 6 to 24 h. The turgor potential of salt-treated cells declined to about zero within 2 h and then slowly recovered to values similar to that of control by 24 h (Fig. 2.6B). Osmotic and turgor potentials of control cells were relatively constant over this 24 h period (Fig. 2.6B).

DISCUSSION

Increases in suspension fresh weight and cell density showed growth of alligator weed cells recovered rapidly after subculture to 200 mol m\(^{-3}\) NaCl (Figs. 2.1A, 2.2). In contrast, the same salinity treatment severely inhibited growth in tobacco suspensions (Fig. 2.1B). Alligator weed and tobacco suspensions were compared because tobacco is a well established cell-suspension system that has been used in a variety of studies that include response to salinity. Previous studies with suspensions from two tobacco varieties showed a 12 to 14 d delay in the growth when exposed to 79 mol m\(^{-3}\) or 171 mol m\(^{-3}\) NaCl (Hasegawa et al., 1980; Heyser and Nabors, 1981; Binzel et al., 1985) which is similar to our results for tobacco (Fig. 2.1B). The lag is shortened to 8 to 10 d when tobacco cultures are grown with 10 \(\mu\)M ABA at 171 mol m\(^{-3}\) NaCl (La Rosa, Hasegawa, Rhodes, Clithero, Wata and Bressan, 1987). The differences in recovery of growth between tobacco and alligator weed are partially due to differences in the ability of cells to initially survive subculture to 200 mol m\(^{-3}\) NaCl. Tobacco cells did not survive the salt treatment as well as alligator weed cells: at 7 d after subculture, tobacco cell viability was still less than 50% while viability of alligator weed cells was 86%
Other species do have growth responses similar to that of alligator weed when subcultured to approximately 200 mol m$^{-3}$ NaCl. After a short lag period, cell density begins to increase in suspension cultures of the monocotyledonous halophytes *Distichlis spicata* (Warren and Gould, 1982) and *Spartina pectinata* (Warren *et al.*, 1985). In other studies of cell cultures from halophytes, the increase in dry weight of *Suaeda maritima* cells is almost equal in control and 180 mol m$^{-3}$ NaCl (von Hedenström and Breckle, 1974), and the halophyte *Atriplex nummularia* maintains 90% cell viability at 2 d after transfer to 400 mol m$^{-3}$ NaCl (Casas *et al.*, 1991). Therefore the response of suspensions from alligator weed, a species not normally found in highly saline environments but capable of withstanding high salinity (Longstreth, *et al.*, 1984) is more like the response of cultures from halophytes than from tobacco.

Both cell fresh weight (Fig. 2.3A) and symplastic volume (Fig. 2.4) showed salt-treated alligator weed cells were smaller than control cells. Salt-treated cells lost substantial volume in the 2 h following subculture (Fig. 2.4 inset). From 2 h to 3 d there was apparent recovery so that symplastic volume in the salt-treated cells was about 11% less than in the control cells (Fig. 2.4). Between 3 d and 7 d the differences between treatments in cell volume remained relatively constant (Fig. 2.4) even though cell density more than doubled (Fig. 2.2). Therefore there are apparently two causes of the reduced volumes of salt-treated cells: first, osmotic loss of water in cells experiencing the change in salinity and later, formation of new cells
with smaller cell volumes than those in control cells. The similarity in cell turgor between the two treatments (Fig. 2.5B) argues that the relationship between final cell volume and turgor was altered for cells that expanded after subculture to high salinity. Such an effect of salinity on these culture cells is consistent with the effect of salinity on the relationship between cell water content and turgor in intact shoots of alligator weed (Bolanos and Longstreth, 1984). Bolanos and Longstreth (1984) found cell wall elasticity (as inferred from the bulk elastic modulus) decreased with increasing salinity, making cells effectively more "rigid" in the face of water uptake or loss. Binzel et al. (1985) previously discussed such changes in cell wall properties with regards to gradual adaptation of tobacco cell cultures to high salinity.

Subculture from MS to 200 mol m\(^{-3}\) NaCl (in MS) was a substantial shock for cellular water balance since the water potential of this salt treatment, -1.30 MPa, was considerably lower than the cell osmotic potential at the time of transfer, -0.60 MPa. This produced a water potential gradient that required water flow out of cells, consistent with the decline in fresh weight during the first 24 h (Fig. 2.3A) and the 36% decline in symplastic volume during the first 2 h after subculture (Fig. 2.4 inset). Symplastic volume then increased (Fig. 2.4 inset) while the osmotic potential decreased further by 6 h (Fig. 2.6A). This suggests salt-treated cells accumulated solutes between 2 and 6 h and this led to sufficient osmotic adjustment for cells to take up water. There was significant turgor at 6 h and a linear increase in turgor potential over the next 18 h (Fig. 2.6B). Osmotic adjustment apparently takes
longer in other species. Osmotic adjustment appears to take 12 h in *D. spicata* when subcultured to 200 mol m$^{-3}$ NaCl (Daines and Gould, 1985). Turgor potential in tobacco cells requires almost 40 h to increase to a stable value after subculture to 86 mol m$^{-3}$ NaCl (Dracup and Greenway, 1988), although this time may be shortened by addition of ABA (La Rosa *et al.*, 1987). In addition, carrot suspension cells require 35 h to adjust to an increase of 150 mol m$^{-3}$ NaCl as evidenced by numbers of plasmolyzed cells (Reuveni, Lerner and Poljakoff-Mayber, 1991).

The time course for osmotic adjustment to a sudden increase in NaCl is not well characterized in higher plant cells. In this study, solutes were initially concentrated in the salt-treated alligator weed cells as a physical consequence of water loss. Salt-treated cells became isosmotic by 2 h and thereafter osmotic potential decreased slowly. The decrease in osmotic potential, increase in turgor potential and partial recovery of symplastic volume after 6 h indicates a mechanism for accumulation of solutes by these cells. Certain unicellular algae appear to adjust to osmotic change by changes in volume. These algae, particularly *Platymonas subcordiformis*, lack cell walls and complete the change in volume within about 10 min after an increase in salinity (Kirst, 1977a). There is also osmotic adjustment in *P. subcordiformis* which involves two phases, first a rapid uptake of inorganic ions during the first 24 h and then a slower phase involving uptake of sugars and synthesis of mannitol (Kirst, 1977b). Further characterization of solute
accumulation by alligator weed is necessary to determine if osmotic adjustment involves different osmotica at different times after subculture to 200 mol m$^{-3}$ NaCl.

LITERATURE CITED


CHAPTER 3

CONCENTRATIONS OF MAJOR SOLUTES IN ALLIGATOR WEED SUSPENSION CELLS AFTER AN ABRUPT INCREASE IN SALINITY

INTRODUCTION

Osmotic adjustment is a net increase in intracellular concentrations of solutes that is independent of the change in volume due to water loss or dehydration (Hsiao, Acevedo and Ferreres, 1976). Solutes that are important for osmotic adjustment of suspension cells from several species have been identified and quantified. Inorganic ions, mainly $\text{Na}^+$ and $\text{Cl}^-$, are accumulated to high concentrations by several plant species during osmotic adjustment (Heyser and Nabors, 1981; Binzel, Hasegawa, Rhodes, Handa, Handa and Bressan, 1987; Sabbah and Tal, 1990; Reuveni, Lerner and Poljakoff-Mayber, 1991; Blits, Cook and Gallagher, 1993). Suspension cells from the halophyte *Distichlis spicata* accumulate proline, an organic solute, when transferred from 0 to 200 mol m$^{-3}$ NaCl (Daines and Gould, 1985). Other organic solutes such as quaternary ammonium compounds, sugars and sugar alcohols have been hypothesized to be important towards osmotic adjustment (Wyn Jones and Gorham, 1983). How the concentration of specific solutes varies over time after cells are exposed to an increase in salinity has not been studied rigorously. Understanding the dynamics of different solute pools after suspension cells are transferred from low to high salinity could provide insight into the control of osmotic adjustment by cells in intact plants experiencing salinity stress.
Suspension cells of alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb), subculutred from 0 to 200 mol m\(^{-3}\) NaCl, exhibit a rapid decline in osmotic potential (\(\psi_\text{s}\)) to the value of the salt treatment medium by 2 h (Chapter 2). By 6 h, the \(\psi_\text{s}\) of salt-treated cells declines more slowly and positive turgor potential (\(\psi_\text{p}\)) is attained. The fresh weight of salt-treated cells starts to increase by 3 d, while cell viability recovers to control levels by 7 d (Chapter 2). In contrast, tobacco cells exposed to the same salt treatment did not exhibit a substantial increase in fresh weight over 21 d nor recovery of cell viability by 7 d (Chapter 2). To further explain these physiological responses to an abrupt increase in salinity by alligator weed and tobacco suspension cells, solute concentrations were determined at different times after salt treatment. The general objective of this chapter was to quantify the solutes that are important for osmotic adjustment by alligator weed cells subjected to an abrupt increase in salinity. The \(\psi_\text{p}\) and inorganic ion concentrations were also determined for salt-treated tobacco cells to extend the previous comparison with alligator weed (Chapter 2).

**MATERIALS AND METHODS**

*Experiments and Culture*

Alligator weed and tobacco suspension cells were maintained and salt treatment was applied to cells as described in Chapter 2. Alligator weed cells were harvested four times during a 24 hour experiment and five times during a
21 day experiment. Tobacco cells were sampled four times during a 14 d experiment. The apoplastic and symplastic volumes of alligator weed and tobacco suspension cells were measured by differential labeling with \(^3\)H\(_2\)O and \(^3\)H-sorbitol as previously described (Chapter 2). Three culture flasks each from the control and salt treatment were harvested at each time point and each experiment was repeated.

**Solute Concentrations**

Cells were harvested by the syringe-barrel method (Chapter 2) with some modifications. Briefly, cells were transferred to syringe barrels lined with two layers of filter paper and washed twice with isotonic solutions of PEG 6000 to remove extracellular and apoplastic solutes which could interfere with the analysis. Two washes with isotonic PEG was sufficient to remove measurable Cl\(^-\) in preliminary experiments. The syringe barrels (with washed cells) were centrifuged at 880 g for 5 min. Cell sap solutions were extracted from the cell pellets by a freeze-thaw method (Chapter 2). The resulting cell sap solutions were used for analysis of several inorganic and organic solutes that could be important to osmotic adjustment. The cell sap solution is a mixture of apoplastic and symplastic solutions (Chapter 2). However, since the apoplastic solutes were eluted by washing with PEG 6000, the intracellular concentrations of solutes reported here were calculated based on values of symplastic volumes.
Inorganic ion concentrations for both alligator weed and tobacco suspension cells were measured in the cell solution and the growth media. \([\text{Na}^+]\) and \([\text{K}^+]\) were measured with an inductively coupled plasma atomic emission mass spectrophotometer (model ARL 34000, LABCO Inc., Dearborn, MI, USA) and \([\text{Cl}^-]\) was measured with an automatic Cl' titrator (AMINCO, Silver Spring, MD, USA).

Concentrations of organic solutes were measured in the cell solutions and growth media from alligator weed. Total reducing sugars were measured using the methods of Nelson (1944). Invertase was used to convert sucrose to reducing sugars which were again measured by the methods of Nelson (1944). Sucrose concentration was taken as the difference between the concentrations of total reducing sugars before and after invertase treatment, divided by 2. Starch concentration was analyzed in the cell pellets obtained after extraction of the cell solutions. Starch in the pellet was hydrolyzed with \(\beta\)-amyloglucosidase using the methods described by Sasek, DeLucia and Strain, (1985). Hydrolyzed starch was then measured using the assay procedures for reducing sugars and was expressed as glucose units.

Proline concentration was measured using the acid-ninhydrin assay (Bates, Walden and Teare, 1973). Quaternary ammonium compounds (QAC) were measured using \(\text{I}_2\text{KI}\) reagent and glycine betaine as standard (Storey and Wyn-Jones, 1977). The concentration of total free amino acids was determined after
Rosen (1957), using leucine as a standard, and malate concentration was measured after Gutman and Wahlefeld (1964). The contribution of each solute to cellular $\psi_c$ was estimated using the Van't Hoff equation taking into account ionic interactions with the Debye-Hückle equation (Nobel, 1991).

**Turgor Potential ($\psi_p$) of Alligator Weed and Tobacco Cells**

The osmolality of cell and media solutions of alligator weed and tobacco suspension cells were measured with a freezing point osmometer (Chapter 2). Osmotic potential ($\psi_o$) was calculated using the formula described previously (Chapter 2). Water potential ($\psi$) of the cells was assumed to be in equilibrium with the growth medium and $\psi_p$ was the difference between $\psi$ and $\psi_c$.

**RESULTS**

**Intracellular Concentration of Solutes in Alligator Weed Cells**

The $[K^+]$ of control cells of alligator weed was at least four-fold greater than the $[Na^+]$ and $[Cl^-]$ throughout 21 d of growth (Fig. 3.1A, B). The concentrations of all three ions were relatively constant over 24 h and thereafter in control cells with $[K^+]$, $[Na^+]$ and $[Cl^-]$ averaging about 80.6 mol m$^{-3}$, 13.3 mol m$^{-3}$ and 11.4 mol m$^{-3}$, respectively. In contrast, $[Na^+]$ and $[Cl^-]$ were 30 and 20 fold higher in salt-treated cells than in control cells by 2 h (Fig. 3.1C). The $[Na^+]$ of salt-treated cells decreased by 42% at 7 d, while $[Cl^-]$ of salt-treated cells decreased by 15% between 3 and 7 d (Fig. 3.1D). The $[Na^+]$ was higher
Figure 3.1. Intracellular concentrations of Na⁺, Cl⁻ and K⁺ ions from control (A and B) and salt-treated cells (C and D) of alligator weed at different times. Each bar represents the mean of three replicates ± s.d.
than [Cl\(^-\)] in salt-treated cells through 3 d (Fig. 3.1C, D). [K\(^+\)] in salt-treated cells was higher than in control cells throughout the experiment (Fig. 3.1).

The concentration of total reducing sugars in salt-treated cells was three times that in control cells at 24 h (Fig. 3.2A, C), but concentrations were similar at 14 d (Fig. 3.2D). The concentration of sucrose increased in salt-treated cells between 3 and 7 d and remained higher than that of control cells throughout 21 d (Fig. 3.2B, D). The malate concentration in salt-treated cells was two times that of the control cells at 12 h and remained higher through 21 d (Fig. 3.2C, D).

The starch concentration of salt-treated cells was lower than that of control cells between 12 h and 3 d (Table 3.1). The starch content of salt-treated cells began to increase between 3 and 7 d and was similar to the value of control cells by 7 d (Table 3.1). The trends were similar regardless of the basis for expression of starch concentration.

The concentration of quaternary ammonium compounds in salt-treated cells increased four-fold within 24 h and remained higher than the control throughout the experiment (Fig. 3.3). The proline concentration was similar in control and salt-treated cells during the first 24 h period and through 3 d (Fig. 3.3A, C), but was higher in salt-treated cells at 7 d and thereafter (Fig. 3.3B, D). The total free amino acid concentrations were higher in salt-treated cells as compared to control cells at 7 d through 21 d (Fig. 3.3 D). The concentration of total free amino acid reported here includes free proline.
Figure 3.2. Intracellular concentrations of total reducing sugars (red. sugars), sucrose and malate from control (A and B) and salt-treated cells (C and D) of alligator weed at different time points. Each bar represents the mean of three replicates ± s.d.
Table 3.1. *Starch concentration in alligator weed cells*

Starch concentration of alligator weed cells expressed on a glucose basis at different times after transfer from 0 to 200 mol m$^{-3}$ NaCl. The values are expressed on the basis of fresh weight (FWt), dry weight (DWt) and per million cells. The values are means of three replicates ($\pm$ s.d). The starch concentration was 16.46 (0.22) mg g$^{-1}$ FWt, 206.01 (2.70) mg g$^{-1}$ DWt, or 1.98 (0.03) mg 10$^6$ cells at day 0.

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Treatment</th>
<th>g$^{-1}$ FWt</th>
<th>Starch Content (mg glucose) g$^{-1}$ DWt</th>
<th>10$^6$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>Control</td>
<td>17.21 (0.31)</td>
<td>215.13 (3.93)</td>
<td>1.97 (0.04)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>15.62 (0.34)</td>
<td>156.25 (3.40)</td>
<td>1.67 (0.04)</td>
</tr>
<tr>
<td>1 d</td>
<td>Control</td>
<td>16.86 (0.19)</td>
<td>210.72 (2.35)</td>
<td>1.93 (0.03)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>15.60 (0.12)</td>
<td>173.33 (1.37)</td>
<td>1.64 (0.02)</td>
</tr>
<tr>
<td>3 d</td>
<td>Control</td>
<td>16.73 (0.47)</td>
<td>196.85 (5.54)</td>
<td>1.87 (0.05)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>16.46 (0.46)</td>
<td>164.55 (4.61)</td>
<td>1.70 (0.05)</td>
</tr>
<tr>
<td>7 d</td>
<td>Control</td>
<td>17.23 (0.46)</td>
<td>191.50 (5.06)</td>
<td>1.99 (0.05)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>18.29 (0.73)</td>
<td>189.96 (7.28)</td>
<td>2.04 (0.08)</td>
</tr>
<tr>
<td>14 d</td>
<td>Control</td>
<td>17.21 (0.23)</td>
<td>191.22 (2.56)</td>
<td>2.02 (0.03)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>18.60 (0.45)</td>
<td>192.60 (4.53)</td>
<td>2.11 (0.05)</td>
</tr>
<tr>
<td>21 d</td>
<td>Control</td>
<td>16.85 (0.16)</td>
<td>186.13 (1.75)</td>
<td>1.96 (0.02)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>18.92 (0.66)</td>
<td>189.21 (6.58)</td>
<td>2.04 (0.07)</td>
</tr>
</tbody>
</table>
Figure 3.3. Intracellular concentrations of quaternary ammonium compounds (QACs), proline and total free amino acids (TFAA) of control (A and B) and salt-treated cells (C and D) of alligator weed at different time points. Each bar represents the mean of three replicates ± s.d.
**Tobacco Response**

The $\psi_p$ of both alligator weed and tobacco cells was essentially zero after 2 h of salt treatment (Fig. 3.4). The $\psi_p$ of alligator weed cells then increased to about 0.18 MPa by 12 h (Fig. 3.4A), but the $\psi_p$ of tobacco cells remained essentially zero through 24 h (Fig. 3.4B). The $\psi_p$ in control cells of alligator weed and tobacco were similar for the first 24 h following transfer (Fig. 3.4).

For tobacco, $[\text{Na}^+]$ and $[\text{Cl}^-]$ were 30 times greater in salt-treated cells than in control cells at all measurement times (Fig. 3.5). The $[\text{K}^+]$ was similar in control and salt-treated tobacco cells at 1 d but by 7 d the $[\text{K}^+]$ in salt-treated cells was 30% lower than that in control cells. The $[\text{Na}^+]$ was similar to the $[\text{Cl}^-]$ of salt-treated tobacco cells throughout the experiment (Fig. 3.5).

**DISCUSSION**

There were large increases in $[\text{Na}^+]$ and $[\text{Cl}^-]$ during the first 24 h of salinity treatment which coincide with the decrease in $\psi_s$ in alligator weed suspension cells (Fig. 2.6A). These three ions accounts for over 50% of the $\psi_s$ of salt-treated cells at 12 h and 1 d (Fig. 3.6A, B). The increases in $[\text{Na}^+]$ and $[\text{Cl}^-]$ were by far the greatest for any of the solutes after an abrupt increase in salinity (Fig. 3.1B, D). The increase in $[\text{Na}^+]$ and $[\text{Cl}^-]$ in salt-treated cells between 0 and 2 h was far in excess of a simple concentrating of solutes due to the decrease in symplastic volume (Fig. 2.4 inset). Based on concentrations of ions at time zero and the decrease in symplastic volume at 2 h, $[\text{Na}^+]$ and $[\text{Cl}^-]$
Figure 3.4. Comparison of changes in $\psi_p$ between control and salt-treated cells of alligator weed (A) and tobacco (B) over a period of 24 h. Each point represents the mean of three replicates ± s.d.
Figure 3.5. Intracellular concentrations of Na\(^+\) (shaded bars), Cl\(^-\) (open bars) and K\(^+\) (hatched bars) ions in control (A) and salt-treated cells (B) of tobacco at different time points over a period of 14 d. Each bar represents the mean and ± s. d.
Figure 3.6. Percent contribution of different solutes to symplastic osmotic potential of control (open bars) and salt-treated cells (shaded bars) of alligator weed at 12 h (A), 1 d (B) and 7 d (C). The different solutes presented are Na⁺, K⁺, Cl⁻, sucrose, total reducing sugars (tot. red. sugars), malate, total free amino acids (TFAA), proline and quaternary ammonium compounds (QAC).
would be expected to be 38 mol m\(^{-3}\), the measured values were 340 mol m\(^{-3}\) for [Na\(^+\)] and 189 mol m\(^{-3}\) for [Cl\(^-\)] (Fig. 3.1A, C). K\(^+\) was the major component of \(\psi_s\) in control cells of alligator weed (Fig. 3.6). K\(^+\) is the major osmotic component of most higher plant cells, with [K\(^+\)] being 100 to 120 mol m\(^{-3}\) in the cytoplasm (Flowers and Lauchli, 1983; Marschner, 1986). These results clearly show that alligator weed cells undergo rapid osmotic adjustment after exposure to an abrupt increase in salinity.

Inorganic ions, particularly Na\(^+\) and Cl\(^-\), are thought to be compartmentalized in the vacuole of salt-adapted cells, so that inhibitory and toxic levels of these ions do not accumulate in the cytoplasm (Wyn Jones and Gorham, 1983). Although this study did not examine the compartmentation of ions directly in alligator weed cells, the recovery of symplastic volume, fresh weight and viability by salt-treated alligator weed cells (Chapter 2) suggests that inorganic ions must have been compartmentalized in the vacuoles of these cells. The compartmentation of ions in salt-adapted cells is thought to involve active transport mechanisms (Wyn Jones and Gorham, 1983; Flowers, 1985). Na\(^+\) accumulation in the vacuole may be due to a Na\(^+\)-H\(^+\) antiporter at the tonoplast of salt-adapted cells. Such an antiporter has been reported to have increased activity in vacuoles isolated from salt-treated cell cultures of sugar beet (Beta vulgaris L.) (Blumwald and Poole, 1987), roots of Atriplex nummularia L. (Hassidim, Braun, Lerner and Reinhold, 1991) and roots of barley (Hordeum
vulgare L.) (Garbarino and Dupont, 1988). A slow channel which conducts both Na\(^+\) and Cl\(^-\) ions across the tonoplast membrane of root cells of salt-treated plants of *Plantago maritima* (Maathuis and Prins, 1990) and leaf cells of salt-treated plants of *Suaeda maritima* was recently characterized (Matthuis, Flowers and Yeo, 1992). Such a channel may prove to be of general importance for ion compartmentation by salt-adapted cells.

\[ [Na^+] \] was 42\% and 22\% higher than \([Cl^-]\) at 24 h and 3 d respectively (Fig. 3.2C, D). An imbalance of \([Na^+]\) and \([Cl^-]\) also occurs in tobacco suspensions gradually adapted to either 171 or 340 mol m\(^{-3}\) NaCl (Binzel *et al.*, 1987) and in a number of halophytes in their saline habitats (Flowers *et al.*, 1977). The imbalance between \([Na^+]\) and \([Cl^-]\) in *Atriplex prostrata* grown in 100 mol m\(^{-3}\) NaCl was suggested to be due to a difference in the rates of uptakes of Na\(^+\) and Cl\(^-\) (Reimann, 1992).

The decrease of \([Na^+]\) and \([Cl^-]\) in salt-treated cells between 1 and 7 d indicates that alligator weed cells are effectively regulating cellular concentrations of these ions during prolonged exposure to high salt. Greenway and Munns (1983) hypothesized that active extrusion of ions particularly Na\(^+\) and Cl\(^-\) and the dilution of ions in expanding daughter cells (after cell division) are major controls of intracellular \([Na^+]\) and therefore mechanisms of tolerance to salinity. Notably, the decrease in \([Na^+]\) and \([Cl^-]\) in salt-treated alligator weed cells coincided with the period of growth and cell division (Fig. 2.2).
In contrast to the decline in [Na+] and [Cl] between 1 and 3 d, [K+] was constant in salt-treated cells from 12 h through 21 d and always higher than [K+] in control cells (Fig. 3.1). K+ may have a significant role in balancing vacuolar and cytoplasmic $\psi_*$ of salt-treated cells of alligator weed (Fig. 3.6). These results also indicate that salt treatment did not inhibit K+ uptake but rather enhanced the process in alligator weed cells. Maintenance of high intracellular [K+] was also reported in salt-adapted cells of alfalfa and Citrus species during prolonged exposure to high salinity (Stavarek and Rains, 1984; Ben-Hayyim, Kafkafi and Neumann-Ganmore, 1987). Maintenance of high [K+] was proposed to be an indicator of salinity tolerance in several species of grasses (Jeschke, 1984).

Reducing sugars could play an important role in rapid osmotic adjustment as indicated by the increase in concentration of total reducing sugars between 2 and 12 h in salt-treated cells (Fig. 3.2 C). Soluble sugars, including reducing sugars and sugar alcohols have been identified as important cytoplasmic solutes in several halophytic species (Briens and Lahrer, 1982). The decrease in starch concentrations in salt-treated cells is an indication that salinity affects carbohydrate metabolism. Starch could have been used as a source of metabolites for synthesis of organic solutes needed for osmotic adjustment, particularly during the first 24 h after salt treatment.

The increase in concentration of quaternary ammonium compounds (QACs) during the first 24 h could be important for osmotic adjustment in
alligator weed cells (Fig. 3.3 C). Concentrations of QACs also increased in intact leaves of alligator weed after an increase in salinity (Su, 1986). QACs, in particular betaines, could act as compatible solutes (Chapter 1). Storey and Wyn Jones (1977) identified a number of species that synthesize large quantities of QAC during osmotic adjustment.

Proline, considered an important compatible solute in some species, did not increase in concentration in salt-treated cells during the first 3 d. Similarly, proline concentration did not increase until 3 d after suspension cells of ice plant (*Mesembryanthemum crystallinum*) were transferred from 0 to 300 mol m⁻³ NaCl (Thomas, De Armond and Bohnert, 1992). In contrast, there was a five-fold increase in proline concentration by 12 h after cells of *D. spicata* were transferred from 0 to 200 mol m⁻³ NaCl (Daines and Gould, 1985). Proline is probably not important for rapid osmotic adjustment to salinity by alligator weed cells, but it is likely important in other species like *D. spicata*.

The concentrations of organic solutes may be low on a total cell volume basis but these solutes could have a significant effect on the $\psi_*$ of the cytoplasm. The relative importance of organic solutes to the $\psi_*$ of the cytoplasm can be estimated by making some assumptions about cell characteristics and the location of solutes. Based on measurements from electron micrographs of salt-treated cells of alligator weed, about 73% of the symplast is occupied by the vacuole and about 27% by the cytoplasm. About 90% of the Na⁺ and Cl⁻ ions are localized
cells of alligator weed, about 73% of the symplast is occupied by the vacuole and about 27% by the cytoplasm. About 90% of the Na$^+$ and Cl$^-$ ions are localized in the vacuole, while K$^+$ is equally distributed between vacuole and cytoplasm, in cells of a number of salt-treated species (Lauchli and Flowers, 1983; Jeschke, 1984). Assuming that this distribution of inorganic solutes hold for alligator weed and that all of the organic solutes (QACs, reducing sugars, proline, sucrose and total free amino acids) were localized in the cytoplasm, the $\psi_*$ of both the cytoplasm and vacuole would be about -1.5 MPa at 24 h after the beginning of salt treatment. This calculated isosmotic condition of the vacuole and the cytoplasm would be consistent with the idea that organic solutes play a significant role in osmotic adjustment.

The response of alligator weed suspension cells to an abrupt increase in salinity was distinct from the response of tobacco suspension cells. Salt-treated cells of alligator weed recovered completely from the initial loss of $\psi_*$ by 24 h while salt-treated tobacco cells did not exhibit significant positive turgor over the same 24 h period (Fig. 3.4). Although both alligator weed and tobacco accumulated similar levels of [Na$^+$] and [Cl$^-$] at 24 h after salt treatment, subsequent change in the concentrations of these two ions differ between the two species. The [Na$^+$] and [Cl$^-$] in alligator weed cells declined between 1 and 7 d (Fig. 3.1), but [Na$^+$] and [Cl$^-$] remained high in tobacco cells throughout the
Osmotic adjustment by alligator weed cells is characterized by a rapid
decline of $\psi_s$ between 0 and 2 h, followed by a slower decrease after 2 h of salt-
treatment (Chapter 2). The present results indicate that the sharp decline in $\psi_s$ of
salt-treated cells by 2 h was due to rapid accumulation of Na$^+$ and Cl$^-$ ions,
simultaneous with increases in [K$^+$], and in concentrations of QACs and reducing
sugars. There were also increases in the concentrations of other organic solutes,
specifically, sucrose, proline and total free amino acids after 3 d of salt
treatment. In combination with higher [K$^+$], increased organic solutes could be
important to cellular $\psi_s$ and osmotic adjustment of salt-treated cells during
prolonged exposure to high salt.

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CHAPTER 4
PROTEIN CONCENTRATIONS AND POLYPEPTIDE PATTERNS IN
RESPONSE TO AN ABRUPT INCREASE IN SALINITY

INTRODUCTION

Salinity can alter qualitatively and quantitatively the synthesis of polypeptides in plants (Hasegawa, Handa and Bressan, 1986; Hanson, Hoffman and Grumet, 1986). At least some of these alterations should be related to the regulation of physiological responses that are important for adaptation to high salinity (Hurkman and Tanaka, 1987). One and two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of root and shoot extracts demonstrated differences between plants grown in low salinity and high salinity (100 to 340 mol m$^{-3}$ NaCl) (Hurkman and Tanaka, 1987; Ramagopal, 1987; Hurkman, Tanaka and DuPont, 1988; Ramagopal, 1988). Detailed differences in polypeptide patterns have also been demonstrated between low and high salinity grown cell cultures derived from several glycophytic species including salt-adapted callus or suspension cells of tobacco (Ericson and Alfinito, 1984; Singh, Handa, Hasegawa and Bressan, 1985; King, Hussey and Turner, 1986), corn, barley and sugarcane (Ramagopal, 1986; Ramagopal, 1988; Ramagopal, 1989) and tomato (Ben-Hayyim, Vaadia, and Williams, 1989). These studies identified polypeptides induced after tissues or cells were adapted to high salt, but differences in polypeptide patterns during the initial stages of adjustment to salinity stress were not considered in detail.
Studies of the early period of adjustment to high salinity have revealed useful information on the roles of a number of polypeptides induced by salinity stress. In *Mesembryanthemum crystallinum*, a species that adjusts to high salinity, a number of "early stress proteins" are synthesized between 12 and 36 h (Bohnert, Ostrem, Cushman, Michalowski, Rickers, Meyer, Derocher, Vernon, Krueger, Vazquez-Moreno, Velten, Hoefner, and Schmitt, 1988). During adaptation to salinity, *M. crystallinum* can also switch from C3 photosynthesis to crassulacean acid metabolism (CAM). However, the enzymes responsible for CAM are induced between 72 and 120 h, significantly later than the "early stress proteins" (Hofner, Vazquez-Moreno, Winter, Bohnert and Schmitt, 1987; Bohnert *et al*., 1988; Ostrem, Olson, Schmitt and Bohnert, 1987; Michalowski, Olson, Piepenbrock, Schmitt and Bohnert, 1989; Cushman, Meyer, Michalowski, Schmitt and Bohnert, 1989). Myo-inositol O-methyl transferase, an enzyme that catalyzes the first step in the biosynthesis of the sugar alcohol pinitol, was upregulated in *M. crystallinum* during the first 30 h of salt treatment (Vernon and Bohnert, 1992a; 1992b). Pinitol is a major solute in *M. crystallinum* growing under salt stress and is considered to be compatible with cytoplasmic function (Paul and Cockburn, 1989). Quantitative and qualitative changes in polypeptide patterns of barley seedlings were observed as early as 1 h after the beginning of salt-treatment (Robinson, Tanaka and Hurkman, 1989). Gulick and Dvorak (1992), identified 11 cDNA clones from roots of salt-treated wheatgrass (*Lophophyrum elongatum*), a salt tolerant relative of wheat (*Triticum*
aestivum), that show enhanced expression between 2 and 24 h after treatment with 250 mol m$^{-3}$ NaCl. These 11 cDNAs are coordinately induced and this has been interpreted to mean that the response to high salinity is determined by several genes producing several polypeptides. These studies clearly show that molecular changes occur at the early stages of response to high salinity.

In the present study, protein concentrations and polypeptide patterns were examined in alligator weed suspension cells from 12 h to 7 d after a transfer from 0 to 200 mol m$^{-3}$ NaCl. Unlike cell suspensions from species generally used for studies of biochemical and molecular changes due to salinity stress, alligator weed cells tolerate an abrupt increase in salinity by rapid recovery of turgor and growth (Chapter 2). Salt-treated cells of alligator weed initially accumulate high levels of [Na$^+$] and [Cl$^-$] and then effectively regulate the concentration of these ions (Chapter 3). Suspension cells of alligator weed could have different mechanisms related to osmotic adjustment than suspension cells from species that have been previously studied. To better understand changes that occur at the protein level in alligator weed cells after transfer from 0 to 200 mol m$^{-3}$ NaCl: (a) protein concentrations of both soluble and membrane fractions were quantified, (b) polypeptide patterns of both soluble and membrane protein fractions were determined by one-dimensional LDS-PAGE, (c) polypeptide patterns produced by salinity stress were compared to the patterns produced by ABA (a plant hormone associated with stress induced proteins, (Skriver and Mundy, 1990), heat shock and chilling stress and (d) the
effect of salinity stress on \textit{in vivo} labeling of cells with $^{35}$S-methionine was determined by one and two dimensional gel electrophoresis.

\textbf{MATERIALS AND METHODS}

\textit{Cell Culture}

Cell suspension cultures of alligator weed were maintained as described previously (Chapter 2). Cell suspensions (20 mLs) not previously exposed to high salt were transferred to 100 mL of Murashige and Skoog (MS) medium (control) or to 100 mL of MS medium with 240 mol m$^{-3}$ NaCl to produce a final concentration of 200 mol m$^{-3}$ NaCl (salt-treatment). Suspension cells were harvested at 12 h, 3 d and 7 d after salt-treatment to obtain an early time point related to osmotic adjustment and later time point related to growth recovery (Chapter 2). Experiments were repeated twice and at each time point each treatment was represented by three replicate flasks.

\textit{Extraction of Protein Fractions}

Cells were harvested by filtering through two layers of Miracloth (Calbiochem Corp., La Jolla, CA). Five g of fresh weight from each treatment were ground in buffer A solution consisting of 250 mol m$^{-3}$ sorbitol, 2.0 mol m$^{-3}$ dithiothreitol (DTT), 5% glycerol, 0.03% phenylmethylsulphonate fluoride (PMSF), 1% polyvinylpyrrolidone (PVP40), 2.0 mol m$^{-3}$ EGTA, 2.0 mol m$^{-3}$ MgSO$_4$, and 10 mol m$^{-3}$ Tris-Mes, pH 7.5, using a Polytron (Kinematica Ag, Luzern, Switzerland) set at speed # 5 for 60 sec. The resulting slurry was filtered through four layers of
cheesecloth to remove intact cells and large cellular debris. The filtrate was centrifuged at 13,000 g for 10 min and the resulting supernatant was referred to as the "soluble fraction" and the pellet referred to as the "membrane fraction". The membrane fraction was solubilized in resuspension buffer B consisting of 250 mol m$^{-3}$ sorbitol, 1.0 mol m$^{-3}$ DTT, 2.5% glycerol and 10 mol m$^{-3}$ Bis-Tris propane (BTP), pH 7.5. Soluble and membrane protein extracts were stored at -70 °C until used for assay. Concentrations of soluble and membrane proteins were determined by the dye-binding method using the Bradford reagent and bovine serum albumin as standard (Bradford, 1976).

**Lithium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (LDS-PAGE)**

To separate the different polypeptides in the soluble and membrane fractions, one-dimensional gel electrophoresis was carried out after the procedures described by Laemmli (1970) with some modifications. Briefly, 10-20% acrylamide gradient running gels were used with 5% acrylamide stacking gels. The upper chamber buffer consist of 50 mol m$^{-3}$ Tris-Glycine with 0.20% LDS and 2 mol m$^{-3}$ Na$_2$EDTA at pH 8.9. Soluble and membrane protein extracts containing approximately 50 µg of protein, were precipitated by addition of 4 volumes of acetone and centrifuged at 8,500 g for 1 min in a microfuge. The resulting pellet was solubilized in 40 µl of buffer A plus 20 µl of a resuspension buffer C consisting of 4 parts of 10% LDS, 2 parts of 60% sucrose, 1 part β-mercaptoethanol, and 1% bromophenol blue (v/v). The solution was mixed vigorously for 30 s using a Vortex mixer, heated for 4 min...
at 90°C and centrifuged for 2 min in the microfuge. The clear supernatant containing approximately 50 μg protein was loaded on the gel and electrophoretically separated by applying a total of 1000 volt-hours at constant voltage. Polypeptide bands were visualized by staining the gel with 0.05% Coomassie brilliant blue R dye. For this analysis, the electrophoresis was repeated at least three times on each sample and only differences consistent for the three gels are reported.

Two Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was based on previous procedures (O'Farrell, 1975), with some modifications. Soluble protein samples were prepared for isoelectric focusing (IEF) by solubilizing the acetone protein precipitate in O'Farrell's lysis buffer. For membrane proteins, phenol protein extraction was used to isolate membrane proteins and pellets were solubilized in O'Farrell lysis buffer for IEF (Hurkman and Tanaka, 1986). Proteins were focused in 10 x 2.5 mm tubes for 20 h at 200 V followed by 2 h at 400 V. IEF gels were equilibrated in resuspension buffer C used for LDS-PAGE for 30 min. The second dimension LDS-PAGE was as described above. The range of pH in the IEF gels was determined by procedures from Dr. E. A. Bray (personal communication). Briefly, a tube gel was loaded with 25 μL of O'Farrell lysis buffer and subjected to IEF along with other tube gels loaded with protein samples. At the end of the IEF run, this blank tube gel was cut into 1 cm pieces starting at the basic end and each 1 cm gel piece was resuspended in 1 mL of 0.01 mol m⁻³ KCl in microfuge tubes, mixed
vigorously in Vortex mixer and allowed to stand for one h. The pH of each gel piece in KCl was determined with a pH meter. Samples were electrophoresed at least three times and only differences which were consistent for all replicates are reported.

**In Vivo Labeling of Proteins**

For protein labeling, cells were filtered by Miracloth under sterile conditions and 2 g (fresh weight) of cells were resuspended in 4 mL of fresh growth medium (MS for control and MS + 200 mol m$^{-3}$ NaCl for salt-treated cells). The cells were incubated for 2 h with 100 µCi of $^{35}$S-methionine (specific activity 1000 Ci mmol$^{-1}$, New England Nuclear-Du Pont, Boston, MA), on a shaker at 125 rpm in the dark. Cells were harvested, immediately frozen in liquid N$_2$ and then extracted in buffer A as before (see Extraction of Protein Fractions). The total cpm in the labeled soluble and membrane protein fractions was determined by counting 5 µL of samples after acetone precipitation and solubilization of the acetone labeled precipitates in a liquid scintillation counter. For one-dimensional LDS-PAGE analysis of *in vivo* labeled proteins, 200,000 cpm were loaded into each lane. For 2D gels, 500,000 cpm were loaded into IEF gels for the first dimension. Gels were stained with Coomassie blue, destained and dried under vacuum. Dried gels were then placed in contact with Kodak XAR-5 film and visualized by autoradiography.
Comparison of Effects of Salt and Other Stress Treatments on Polypeptide Patterns

The change in polypeptide patterns of alligator weed after 12 h of salt treatment was compared to the effects of ABA, heat shock and chilling stress to determine the specificity of the changes in polypeptide pattern caused by salt treatment. The treatments used here for ABA, heat shock and chilling stress were based on treatments that produced responses in other species. To determine the effect of ABA, cells were grown in MS media supplemented with 10 mmol m$^{-3}$ ABA (Calbiochem, La Jolla, CA) for 12 h (Singh, LaRosa, Handa, Hasegawa and Bressan, 1987). For heat-shock, cells were grown in MS media at 25 °C for 8 h and at 40 °C for 4 h (Harrington and Alm, 1988). For chilling stress, cells were grown for 6 h at 25 °C and at 4 °C for 6 h (Xin and Li, 1992). Five g fresh weight of cells from each treatment were harvested and both membrane and soluble fractions were isolated as described above. Soluble and membrane proteins were separated by one-dimensional LDS-PAGE and stained with Coomassie blue.

RESULTS

Soluble and Membrane Protein Concentrations

The concentration of soluble protein on a fresh weight basis was 10% lower in salt-treated than in control cells at 12 h after salt-treatment. At 3 and 7 d however, salt-treated cells had 35 and 45% higher soluble protein concentrations on a fresh weight basis than did control cells (Table 4.1). When soluble protein
Table 4.1.  *Concentrations of proteins in the soluble fraction*

Protein concentrations in the soluble fractions from control and salt-treated cells of alligator weed at 12 h, 3 and 7 d after transfer from 0 to 200 mol m\(^{-3}\) NaCl. Protein concentrations were expressed based on per g fresh weight, g dry weight and \(10^6\) cells. The initial values of protein concentration were 2.39 (±0.36) mg g\(^{-1}\) FWt, 26.58 (±4.04) mg g\(^{-1}\) DWt and 0.29 (±0.04) mg \(10^6\) cells. The values presented are mean of three replicates (± s.d).

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Treatment</th>
<th>Protein Concentration (\text{mg g}^{-1}\text{FWt})</th>
<th>Protein Concentration (\text{mg g}^{-1}\text{DWt})</th>
<th>Protein Concentration (\text{mg} 10^6\text{cells})</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td>Control</td>
<td>2.85 (0.24)</td>
<td>28.70 (2.64)</td>
<td>0.32 (0.03)</td>
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<tr>
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<td>Salt</td>
<td>2.58 (0.06)</td>
<td>26.16 (0.49)</td>
<td>0.30 (0.01)</td>
</tr>
<tr>
<td>3 d</td>
<td>Control</td>
<td>2.45 (0.15)</td>
<td>27.26 (1.67)</td>
<td>0.31 (0.02)</td>
</tr>
<tr>
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<td>Salt</td>
<td>3.30 (0.10)</td>
<td>27.53 (0.83)</td>
<td>0.31 (0.01)</td>
</tr>
<tr>
<td>7 d</td>
<td>Control</td>
<td>2.41 (0.11)</td>
<td>24.06 (1.14)</td>
<td>0.31 (0.01)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>3.50 (0.15)</td>
<td>26.93 (1.13)</td>
<td>0.34 (0.01)</td>
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concentration was expressed per g dry weight or per million cells, salt-treated cells and control cells had similar concentrations at all sampling times (Table 4.1).

The concentration of membrane proteins from salt-treated cells was similar to that of control cells at 12 h and 3 d (Table 4.2). At 7 d, the concentration of membrane proteins per g of fresh or dry weight was higher in salt-treated cells than in control cells, but there was no difference on a cell basis.

*Polypeptide Patterns in One-Dimensional Gels*

For both soluble and membrane fractions there were three types of differences between the polypeptide profiles of salt-treated cells and control cells (Figs. 4.1 and 4.2). These include: (a) darker staining of several polypeptide bands from salt-treated cells as compared to control cells at all sampling times, (b) lighter staining of several polypeptide bands from salt-treated cells as compared to control cells at all sampling times and (c) lighter staining of certain polypeptide bands from salt-treated cells as compared to control cells at 12 h and 3 d, but by 7 d these bands were similarly stained in salt-treated and control cells. Five polypeptide bands with M, of 16, 21, 26, 30 and 33 kD were more darkly stained in the soluble fraction of salt-treated cells than in the soluble fraction of control cells (filled arrowheads, Fig. 4.1). In contrast, a 56 kD polypeptide band was less stained in the soluble fraction from salt-treated cells than from control cells at all sampling times (open arrowheads, Fig. 4.1).
Table 4.2.  *Concentrations of proteins in the membrane fractions*

Protein concentrations in the membrane fractions from control and salt-treated cells of alligator weed at 12 h, 3 d and 7 d after transfer from 0 to 200 mol m$^{-3}$ NaCl. Protein concentrations were expressed based on per g of fresh weight, g of dry weight and 10$^6$ cells. The initial values of protein content expressed based on three different parameters mentioned are 0.50 (±0.36) mg g$^{-1}$ FWt, 5.54 (±0.46) mg g$^{-1}$ DWt and 0.06 (±0.01) mg 10$^6$ cells. The values presented are mean of three replicates ± s.d.

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Treatment</th>
<th>Protein Concentration</th>
<th>mg 10$^6$cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg g$^{-1}$FWt</td>
<td>mg g$^{-1}$DWt</td>
</tr>
<tr>
<td>12 h</td>
<td>Control</td>
<td>0.54 (0.04)</td>
<td>5.54 (0.46)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>0.48 (0.07)</td>
<td>6.47 (0.82)</td>
</tr>
<tr>
<td>3 d</td>
<td>Control</td>
<td>0.68 (0.08)</td>
<td>7.54 (0.86)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>0.72 (0.04)</td>
<td>6.57 (0.31)</td>
</tr>
<tr>
<td>7d</td>
<td>Control</td>
<td>0.61 (0.14)</td>
<td>6.07 (1.41)</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>0.81 (0.01)</td>
<td>6.24 (0.11)</td>
</tr>
</tbody>
</table>
Figure 4.1. LDS-PAGE of the soluble protein fraction from control and salt-treated cells of alligator weed. Equal amounts of protein (50 μg) were loaded in each lane. Molecular weight markers are in lane 1. Lanes 2, 3, 5 and 7 are samples from control cells at 0, 12 h, 3 and 7 d respectively. Lanes 4, 6 and 8 are samples from salt-treated cells at 12 h, 3 and 7 d respectively, after transfer to high salt. Filled arrowheads represent polypeptide bands with darker staining intensity in salt-treated cells, while open arrowheads represent protein bands with lighter staining intensity in salt-treated cells.
Figure 4.2. LDS-PAGE of the membrane protein fraction from control and salt-treated cells of alligator weed. Equal amounts of protein (50 μg) were loaded in each lane. Molecular weight markers are in lane 1. Lanes 2, 3, 5 and 7 are samples from control cells at 0, 12 h, 3 and 7 d respectively. Lanes 4, 6 and 8 are samples from salt-treated cells at 12 h, 3 and 7 d respectively, after transfer to high salt. Filled arrowheads represent polypeptide bands with darker staining intensity in salt-treated cells, while open arrowheads represent protein bands with lighter staining intensity in salt-treated cells.
In the membrane fraction, an 18 kD polypeptide band was stained darker and a 28 kD band was stained lighter in protein profiles from salt-treated cells as compared to control at all sampling times (Fig. 4.2). A 35 kD polypeptide band from the membrane fraction of salt-treated cells was stained less at 12 h and 3 d, but by 7 d the staining intensity was similar in the protein profiles from both salt-treated and control cells (Fig. 4.2).

**Protein Synthesis**

In the soluble fraction, polypeptides with M, of 16, 21 and 30 kD, showed more labeling by $^{35}$S-methionine in salt-treated cells than in control cells at 12 h and 3 d (Fig. 4.3). At 7 d however, only the 21 and 30 kD bands had higher label in salt-treated cells as compared to control cells.

In the membrane fraction, 39 and 48 kD polypeptide bands were highly labeled in polypeptide profile of salt-treated cells and were absent in control cells at all sampling times (Fig. 4.4). In contrast, a 27 kD band was labeled less in salt-treated cells than in control cells (Fig. 4.4). An 80 kD polypeptide was labeled less in salt-treated cells at 12 h and 3 d than in control cells, but at 7 d labeling of this band appeared to be similar in both control and salt-treated cells (Fig. 4.4).

**Two Dimensional Gel Electrophoresis**

Three types of differences were observed between the two-dimensional polypeptide profiles of $^{35}$S-methionine labeled salt-treated and control cells: (a) several polypeptide spots present in salt-treated cells were absent in control cells, (b)
Figure 4.3. Autoradiogram of soluble proteins from control and salt-treated cells of alligator weed labeled with \(^{35}\)S-methionine. Equal cpm (200,000) were loaded in each lane. Lanes 1, 2, 4 and 6 represent the protein profiles of control cells at 0, 12 h, 3 and 7 d respectively as indicated. Lanes 3, 5 and 7 represent protein profiles of salt-treated cells at 12 h, 3 and 7 d after salt treatment. Polypeptide bands that showed enhanced labeling in salt-treated cells as compared to control are indicated by filled arrow heads. Numbers on the left represent the position of molecular weight markers.
Figure 4.4. Autoradiogram of membrane proteins from control and salt-treated cells of alligator weed labeled with $^{35}$S-methionine. Equal cpm (200,000) were loaded in each lane. Lanes 1, 2, 4 and 6 represent the protein profiles of control cells at 0, 12 h, 3 and 7 d respectively as indicated. Lanes 3, 5 and 7 represent protein profiles of salt-treated cells at 12 h, 3 and 7 d after salt treatment. Polypeptide bands that showed enhanced labeling are indicated by filled arrow heads, while open arrow heads refer to bands which showed a decrease in labeling in salt-treated cells as compared to control. Numbers on the left represent the position of molecular weight markers.
several polypeptides showed greater labeling in salt-treated cells than in control cells and (c) several polypeptides showed less label in salt-treated cells as compared to control cells. These differences were found for both soluble and membrane fractions.

The labeling of 14 polypeptide spots was different in the soluble fraction of salt-treated cells and control cells (Fig. 4.5 and Table 4.3). Three polypeptides with Mr of 26, 36 and 39 kD and pI's of 4.2, 6.4 and 7.3 respectively, were only found in salt-treated cells (Fig. 4.5B and Table 4.3). Six polypeptide spots were labeled more in salt-treated cells, while five polypeptide spots were labeled less in salt-treated cells (Fig. 4.5B).

The labeling of 15 protein spots was different in the membrane fractions from salt-treated cells and control cells (Fig. 4.6 and Table 4.4). Six polypeptides were unique to salt-treated cells only. Seven polypeptides were labeled more and two polypeptides were labeled less in salt-treated cells than in control cells (Fig. 4.6B and Table 4.4).

**Specificity of Salinity Effects on Polypeptide Patterns**

The polypeptide profiles of control and salt-treated cells at 12 h were compared to the polypeptide profiles of cells exposed to 10 mmol m⁻³ ABA treatment for 12 h, heat shock at 40°C for 4 h and chilling stress at 4°C for 6 h applied right before protein extraction. In the soluble fraction, two polypeptide bands (Mr of 16 and 33 kD) exhibited darker staining in both salt and ABA treated-
Figure 4.5. Two dimensional gel electrophoretic patterns of the soluble fraction of $^{35}$S-methionine labeled proteins from control (A) and salt-treated (B) cells of alligator weed at 3 d after salt treatment. The proteins were separated in the first dimension by isoelectric focusing at pH 3.5-7.5 and in the second dimension by LDS-PAGE. Polypeptide spots which were unique to salt-treated cells are indicated by boxes with arrows, while polypeptides which showed enhanced labeling in salt-treated cells are indicated by boxes only, and circles indicate proteins that decrease in labeling in salt-treated cells as compared to control. The numbers on the left indicate position of molecular weight markers.
Table 4.3. *Differences in two dimensional gel profile of the soluble fraction*

Summary of changes in two-dimensional polypeptide pattern of $^{35}$S-methionine labeled soluble fraction at 3 d after salt-treatment. The molecular weight ($M_r$) and isoelectric point (pI) of polypeptides spots that change in salt-treated cells compared to control as shown in Fig. 4.5 are given. Change in intensity in protein spots between control and salt-treated were scored visually.

<table>
<thead>
<tr>
<th>Types of Change</th>
<th>$M_r(\times 1000)$</th>
<th>pI</th>
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</thead>
<tbody>
<tr>
<td>i) Unique to salt-treated cells</td>
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<tr>
<td></td>
<td>26</td>
<td>4.2</td>
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<td></td>
<td>36</td>
<td>6.4</td>
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<td></td>
<td>39</td>
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<td>ii) Increased in salt-treated cells</td>
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<tr>
<td></td>
<td>14</td>
<td>6.2</td>
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<td></td>
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<td>4.7</td>
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<tr>
<td></td>
<td>29</td>
<td>4.9</td>
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<td>iii) Decreased in salt-treated cells</td>
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<tr>
<td></td>
<td>46</td>
<td>6.8</td>
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<tr>
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<td>80</td>
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Figure 4.6. Two dimensional gel electrophoretic patterns of the membrane fraction of $^{35}$S- methionine labeled polypeptides from control (A) and salt-treated (B) cells of alligator weed at 3 d after salt treatment. The proteins were separated in the first dimension by isoelectric focusing at pH 3.5-7.5 and in the second dimension by LDS-PAGE. Polypeptide spots which were unique to salt-treated cells are indicated by boxes with arrows, while polypeptides which showed enhanced labeling in salt-treated cells are indicated by boxes only, and circles indicate proteins that decrease in labeling in salt-treated cells as compared to control. The numbers on the left indicate the position of the molecular weight markers.
Table 4.4.  *Differences in two dimensional gel profile of the membrane fraction*

Summary of changes in two-dimensional polypeptide pattern of $^{35}$S-methionine labeled membrane fraction at 3 d after salt-treatment. The molecular weight (M$_r$) and isoelectric point (pI) of polypeptides spots that change in salt-treated cells compared to control as shown in Fig. 4.6 are given. Change in protein spots between control and salt-treated was scored visually.

<table>
<thead>
<tr>
<th>Types of Change</th>
<th>M$_r$(x1000)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Unique to salt-treated cells</td>
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<tr>
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<td>44</td>
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<tr>
<td>ii) Increased in salt-treated cells</td>
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</tr>
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<tr>
<td>iii) Decreased in salt-treated cells</td>
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cells as compared to control cells (Fig. 4.7). The polypeptide profile of soluble fraction from heat shocked and chilling stressed cells were more similar to that of control cells (Fig. 4.7). The changes observed in the polypeptide profile from the membrane fraction of salt-treated cells as compared to control cells were not detected from the other treatments (Fig. 4.8). The polypeptide profile of the membrane fraction from ABA treated, heat shocked and chilling stressed cells were similar to that of the control (Fig. 4.8).

DISCUSSION

A transfer of alligator weed suspension cells from 0 to 200 mol m⁻³ NaCl does not appear to result in significant inhibition of protein synthesis since the protein concentrations per cell were similar between control and salt-treated cells at all sampling times and differences of concentration based on other parameters were small (Tables 4.1 and 4.2). However, there were differences in staining intensity of several polypeptides from the soluble and membrane fractions of alligator weed suspension cells as early as 12 h after a transfer from 0 to 200 mol m⁻³ NaCl (Figs. 4.1 and 4.2). Five out of 33 polypeptide bands in the soluble fraction and one polypeptide band out of 36 polypeptides in the membrane fraction were more intensely stained in salt-treated cells than in control cell at all sampling times. In contrast, one polypeptide band in the soluble fraction and two polypeptide bands in the membrane fraction were stained less in salt-treated cells than in control cells. These differences between treatments indicate that a number of relatively abundant
Figure 4.7. Comparison of the effect of high salinity to ABA treatment, heat shock and chilling stress on LDS-PAGE polypeptide patterns of the soluble fraction of alligator weed. Lanes are: 1-control; 2- salt treatment for 12 h; 3- ABA treatment; 4- heat shock and 5- chilling stress. Filled arrowheads represent polypeptide bands with darker staining intensity in salt-treated cells, while open arrowheads represent protein bands with lighter staining intensity in salt-treated cells. Numbers on the left indicate positions of molecular weight markers.
Figure 4.8. Comparison of the effect of high salinity to ABA treatment, heat shock and chilling stress on LDS-PAGE polypeptide patterns of the membrane fraction of alligator weed. Lanes are: 1-control; 2- salt treatment for 12 h; 3- ABA treatment; 4- heat shock and 5- chilling stress. Filled arrowheads represent polypeptide bands with darker staining intensity in salt-treated cells, while open arrowheads represent protein bands with lighter staining intensity in salt-treated cells. Numbers on the left indicate positions of molecular weight markers.
proteins were affected by an abrupt increase in salinity. This has been observed in other salinity studies. For example, osmotin, a 26 kD polypeptide, accounts for about 12% of the total cellular protein concentration in salt adapted tobacco suspension cells (Singh et al., 1985, 1987).

*In vivo* labeling of proteins with $^{35}$S-methionine showed quantitative and qualitative differences between control and salt-treated cells in the synthesis of soluble and membrane proteins (Figs. 4.3 and 4.4). Salinity treatment resulted in increased synthesis of constitutive soluble polypeptides as evidenced by greater labeling of four polypeptide bands in the soluble fraction of salt-treated cells (Fig. 4.3). The increase in label in these polypeptides at 12 h coincided with osmotic adjustment, thus raising the possibility that these polypeptides are related to the process of osmotic adjustment in alligator weed cells. Differential synthesis of a number of proteins occurs during the early stages of exposure to salinity in *L. elongatum* and *M. crystallinum* (Gulick and Dvorak, 1992; Vernon and Bohnert, 1992). The $M_r$ of induced polypeptides (21 and 30 kD bands) from the soluble fraction of alligator weed were similar to the $M_r$ of some of the enhanced polypeptides identified in salt-adapted cell lines of tobacco (Ericson and Alfinito, 1984; Singh, Handa, Hasegawa and Bressan, 1985; King, Hussey and Turner, 1986). These results suggest that salinity stress possibly affects similar biochemical pathways in different species.
In the membrane fraction of cells labeled with $^{35}$S-methionine, two polypeptides (39 and 48 kD) present in salt-treated cells were absent from control cells (Fig. 4.4). These two polypeptides were present in the membrane fraction of salt-treated cells at 12 h through 7 d. Synthesis of novel membrane polypeptides occurs in salt-treated seedlings of barley (Hurkman and Tanaka, 1987; Hurkman, Tanaka and DuPont, 1988). Although several studies have examined the changes in polypeptide patterns during salinity stress using cell cultures, this is the first documented report of unique membrane polypeptides associated with salinity stress of suspension cells.

There were differences between the results obtained from the Coomassie blue stained gels and autoradiograms of both soluble and membrane fractions. In particular, only three of the five polypeptides identified from the stained gel of the soluble fraction of salt-treated cells exhibited increased incorporation of label in the autoradiogram (Figs. 4.1 and 4.3). Also, the two membrane polypeptides that were induced in the autoradiogram of salt-treated cells were not detected in the Coomassie blue stained gel (Figs. 4.2 and 4.4). These differences could be due to at least two possible reasons. First, polypeptides detected in Coomassie blue stained gels represent the steady-state level of proteins present in the cells and do not indicate differences in rates of synthesis and turnover. However, the autoradiogram basically shows synthesis during the labeling period only. Secondly, *in vivo* labeling is specific, for this case labeling with $^{35}$S-methionine emphasized the expression of
methionine-rich polypeptides. It is possible that some of the polypeptides that increased in the soluble fraction have lower methionine content.

Polypeptide profiles from two dimensional gel electrophoresis provided information on the isoelectric point of polypeptides that increased at 3 d after the beginning of salt treatment (Table 4.3 and Table 4.4). Polypeptides spots that were different in labeling intensity between the two treatments that were not found in one dimensional LDS-PAGE gels were detected in two-dimensional gel. These multiple differences between control and salt-treated cells probably reflect the higher sensitivity of the two dimensional gel separation method which can detect even subtle modifications in amino acid composition of polypeptides (O'Farrell, 1975). Bohnert et al., (1988) reported that by two dimensional gel electrophoresis, 30 polypeptide spots were different between control and salt-treated plants of *M. crystallinum* at 3 d after salt treatment. Differences in 10 polypeptide spots were resolved between two dimensional gel patterns of salt-adapted and unadapted tomato suspension cells (Ben-Hayyim et al., 1989). In sugarcane suspensions, differences in labeling intensity of 15 polypeptide spots between salt-treated and control cells were identified by two dimensional gel electrophoresis (Ramagopal and Carr, 1991).

ABA treatment resulted in enhanced staining of two polypeptides from the soluble fraction with similar *M* to those enhanced by salt treatment (Fig. 4.7). This result was different from effect of ABA on tobacco cells where increased synthesis of polypeptides was detected after radioactive labeling (Singh et al., 1987). This
suggests that ABA could be involved in the response to an abrupt increase in salinity in alligator weed cells. ABA may serve as a signal for the cells to perceive changes in ionic and osmotic conditions (Skriver and Mundy, 1990). The possible involvement of ABA in the physiological responses to salinity stress at the molecular level has been discussed for cell cultures of tobacco and whole plants from various species (Singh, Nelson, Kuhn, Hasegawa and Bressan, 1989; Mundy and Chua, 1989; Galvez, Gulick and Dvorak, 1993). The response of alligator weed cells to high salinity was distinct from the response to heat shock and chilling stress (Figs. 4.7 and 4.8). Heat shock and chilling did not exhibit clear changes in polypeptide profile from that of the control because the time needed for accumulation of polypeptide that change due to these treatments could be longer than that for salt treatment. However, previous studies using in vivo labeling in tobacco cells have shown differences between polypeptide patterns observed from high salt treatment and heat shock cells (Harrington and Alm, 1988). There was a decrease in synthesis of several polypeptides and induction of a new set of polypeptides in heat-shocked tobacco cells (Harrington and Alm, 1988). Chilling stress has also been shown to have a different effect on the polypeptide profiles of rice plants compared to that of salinity treatment based on radiolabeled protein profiles (Hahn and Walbot, 1989).

Transfer of alligator weed cells from 0 to 200 mol m⁻³ NaCl resulted in increased incorporation of label into three soluble polypeptides and induction of two new membrane polypeptides as early as 12 h. These changes in the polypeptide
pattern may be indicative of the changes in protein synthesis that could be associated
with osmotic adjustment to an abrupt increase in salinity in alligator weed cells.

Further studies are needed to elucidate the nature of the relationship between these
induced polypeptides and osmotic adjustment.

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CHAPTER 5
SUMMARY AND CONCLUSIONS

Alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb), is a dicotyledonous C3 species that tolerates fluctuating levels of salinity. Suspension cells of alligator weed were used in this study to investigate physiological responses to an abrupt increase in salinity (0 to 200 mol m\(^{-3}\) NaCl). The first goal of this work was to characterize the growth and water relations of alligator weed suspension cells after an abrupt increase in salinity and to compare the response of alligator weed to that of tobacco. The second goal of this work was to quantify the concentrations of solutes that could be important for osmotic adjustment by alligator weed suspension cells. The third goal of this study was to analyze protein concentrations and polypeptide patterns of alligator weed suspension cells after an abrupt increase in salinity.

Stable, dark-grown suspension cell cultures were developed from alligator weed leaves for this study. The principal results from this study are summarized in Fig. 5.1. This figure shows 10 parameters that appears to be most tightly linked with alligator weed response to an abrupt increase in salinity.

Increasing fresh weight of salt-treated cells at 3 d indicated a rapid recovery of growth by alligator weed cells after exposure to an abrupt increase in salinity (Fig. 5.1A). The fresh weight of salt-treated cells more than doubled by 7 d. Although the fresh weight of salt-treated cells was lower than that of control cells,
Summary of changes in relative values of 10 parameters evaluated in this study during the first 168 h. The relative values shown here are for salt-treated cells expressed as a percent of control at each time point. The different parameters were grouped according to the magnitude of values to emphasize changes with time. Abbreviations are: FWt-fresh weight, SymV-symplastic volume, QAC-quaternary ammonium compounds, Red Sugars-reducing sugars and Pro-proline.
alligator weed clearly survived and adapted to an abrupt increase in salinity. This response was correlated with similar patterns of increasing cell density and cell viability (Chapter 2).

At 2 h there was a decline in cellular osmotic potential \( \psi_c \) of salt-treated cells to the value of the \( \psi_c \) of the salt-treatment medium (Chapter 2), concomitant with a decrease in symplastic volume (Fig. 5.1A). Therefore, an abrupt increase in salinity initially resulted in water loss, but cell contents became isosmotic with the media by 2 h. The decrease in \( \psi_c \) coincided with large increases in intracellular \([Na^+]\) and \([Cl^-]\) in salt-treated cells by 2 h (Fig. 5.1B). Similarly, \([K^+]\), reducing sugars and quaternary ammonium compounds also increased in salt-treated cells by 2 h (Fig. 5.1B). Although Na\(^+\) and Cl\(^-\) ions play a primary role in osmotic adjustment of alligator weed cells, the organic solutes could also be important, particularly if these solutes were localized in the cytoplasm.

From 2 to 6 h, there was continued but slower decline in \( \psi_c \), partial recovery of symplastic volume and development of substantial positive turgor potential \( \psi_t \) (Fig. 5.1A). By 3 d after salt treatment began, \( \psi_t \) had recovered to control values, which correlated with the increase in fresh weight. These results indicate that there was full recovery of salt-treated cells leading to resumption of growth.

At 7 d, concentrations of proline, sucrose and total free amino acids increased and \([K^+]\), higher than that found in control cells, was maintained (Fig. 5.1C, D). These changes in organic solute concentrations, which are different from
those that occur during the first 24 h of salt treatment, and the decrease in [Na\(^+\)] and [Cl\(^-\)] from 1 to 7 d indicate a shift in cell response as salinity is prolonged.

The physiological responses of alligator weed cells to an abrupt increase in salinity were distinct from responses of tobacco cells, a well-studied glycophyte. Fresh weight and cell density of salt-treated alligator weed cells more than doubled by 7 d but the fresh weight of salt-treated tobacco suspensions did not double during the 21 d experiment (Chapter 2). Cell viability dropped from about 90% to 77% in alligator weed and to 42% in tobacco at 1 d and remained low (47%) in tobacco at 7 d (Chapter 2). The \(\psi_p\) of salt-treated cells of alligator weed was clearly positive by 6 h (Fig. 5.1A; Chapter 2) but \(\psi_p\) of salt-treated tobacco cells was essentially zero through 24 h (Chapter 3). [Na\(^+\)] and [Cl\(^-\)] increased in tobacco cells and remained high for 14 d after subculture to 200 mol m\(^{-3}\) NaCl, while concentrations of these ions declined by 7 d in salt-treated cells of alligator weed (Chapter 3). [K\(^+\)] declined in tobacco between 1 and 7 d, while it was maintained at a high level in salt-treated cells of alligator weed (Chapter 3). These differences suggest that the physiological responses of alligator weed, a species not normally found in highly saline environments, but one that tolerates increases in salinity are more like the response of halophytes than that of a glycophyte like tobacco.

Distinct differences in the polypeptide patterns of control and salt-treated alligator weed cells indicate that physiological responses to an abrupt increase in salinity involve alterations at the molecular level. There was increased incorporation
of radioactive label into two polypeptides in the soluble fraction from cells at 12 h to 7 d of salt treatment (Chapter 4). In the membrane fraction two new membrane polypeptides were detected (Chapter 4). These results suggest that there are polypeptides that are preferentially synthesized during osmotic adjustment and they may play a role in the initial response of alligator weed cells to an abrupt increase in salinity.

This study identified cellular responses that were important for adaptation to an abrupt increase in salinity. The comparison of alligator weed and tobacco showed that rapid recovery of positive turgor was critical to adaptation to high salinity. Salt-treated cells of alligator weed had positive $\psi_p$ by 6 h, while $\psi_p$ in salt-treated tobacco cells was still about zero at 24 h. Furthermore, the ability to regulate $[Na^+]$ and $[Cl^-]$ appeared significant for greater tolerance of alligator weed cells to high salinity. Both of these differences could be related to specialized features of cell membranes in alligator weed cells. This work also presented a different approach for investigating adaptation and tolerance to high salinity at the cellular level. The early adjustment by alligator weed cells not previously exposed to high salt, particularly in the first 24 h, indicates the inherent capability of alligator weed cells for adaptation to high salinity. The response of alligator weed cells to continued exposure to salinity (after 24 h), could be due to selection of more salt-tolerant cells in the population since cell division was occurring after that time.
These results also suggest further studies using other physiological parameters to better understand the responses of alligator weed to high salinity. Measurements of influx and efflux rates of radioisotopes of Na⁺, Cl⁻ and K⁺ could clarify whether salt-treated cells are extruding ions from all cells or excluding them from cells arising after the beginning of high salt treatment. Metabolic adjustment by alligator weed cells during salinity treatment also requires further attention. Changes in carbohydrate pools indicate that an important focus might be the evaluation of the key enzymes in sugar metabolism. Analysis of the synthetic pathways for the organic solutes that changed in concentration in response to salinity would also be important for better understanding of osmotic adjustment in alligator weed. There is also a need for radiolabeling studies to clarify the relationship between response to salinity and the response to ABA treatment, heat shock and chilling stress. Information from this radiolabeling study will be useful towards identification of proteins specifically related to the response to salinity stress.
APPENDIX

CALLUS INITIATION AND ESTABLISHMENT OF SUSPENSION CELL CULTURES FROM ALLIGATOR WEED LEAVES

INTRODUCTION

This appendix describes the initiation of callus and subsequent development of suspension cell cultures from leaf tissue of alligator weed.

MATERIALS AND METHODS

Calli were initiated from the 2nd to the 4th leaves beneath the stem apex of alligator weed plants grown in hydroponic culture in a growth chamber. Leaves were immediately washed with deionized water and blotted dry, and all subsequent steps were carried out under aseptic conditions in a laminar flow hood. Leaves were surface sterilized by soaking in 70% ethanol for 30 s, then in 10% sodium hypochlorite solution for 20 min and rinsing three times with sterile distilled water. The leaves were then transferred to sterile petri dishes lined with moist filter paper and 0.5 cm diameter leaf discs were obtained from leaf blades.

Three leaf discs were cultured in a petri dish containing 20 mLs of MS growth medium. This MS medium was composed of macronutrients and micronutrients defined by Murashige and Skoog (1962), and was supplemented with vitamins defined by Gamborg, Miller and Ojima (1968), sucrose at 30 g L⁻¹, 2, 4-dichlorophenoxyacetic acid (2,4-D) at 1 mg L⁻¹ and bactoagar at 8 g L⁻¹. The pH of this medium was adjusted to 5.7. The petri dishes with alligator weed leaf explants on MS medium were covered tightly with parafilm and were maintained in
the dark at 25 °C. Leaf calli were allowed to develop and then transferred to fresh medium every 30 d for three passages. Only cultures with yellow-green masses of cells were used for subsequent transfer. Brown or black cultures were discarded.

After four transfers, the soft mass of cells on the outer periphery of the callus (also referred to as friable cells) were isolated from the harder and more compact core of the callus. These two groups of cells were transferred to MS liquid growth medium (3 g of cells in MS medium without bactoagar) to determine which produce better source of suspension cultures. Liquid cultures were maintained in the dark on a rotary shaker set at 125 rpm and 25 °C. The physical appearance of suspension cells were examined every other day for 21 d. The volume of samples needed for transfer and period between transfers were also optimized.

RESULTS AND DISCUSSION

Undifferentiated cell masses started to develop from alligator weed leaves after 14 d on solid MS medium. Callus cells grew slowly during the first 30 d but grew rapidly after two transfers. Callus initiation on 2,4-D (a synthetic auxin-like plant growth hormone) followed similar pattern in a number of species (Wareing and Phillips, 1986).

Suspension cell cultures derived from the soft friable calli had whitish color, and cells were well dispersed throughout the medium. In contrast, suspension from the hard core calli were brown and cell density was obviously lower than that of suspension from the friable calli after 21 d. Cells from core calli turned brown and
remained intact in the suspension despite continuous shaking. The best and most consistent growth occurred with transfer of 10 mLs of cell suspension to 100 mLs of fresh MS media every 7 d. Cell clumping was minimized by passing the suspension through autoclavable tubes fitted with a 4 mm$^2$ nylon mesh every 10 cycles of transfer.

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VITA

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Major Field: Botany

Title of Dissertation: Characterization of Physiological Responses of Suspension Cells from Alligator Weed (Alternanthera philoxeroides [Mart.] Griseb) to an Abrupt Increase in Salinity

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Date of Examination:

November 3, 1993