DISSERTATION

PHYSICAL AND CHEMICAL ALTERATION OF *Distichlis spicata* L. CELL WALLS WITH NaCl AND WATER STRESS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION "PHYSICAL AND CHEMICAL ALTERATION OF *Distichlis spicata* L. CELL WALLS WITH NaCl AND WATER STRESS" PREPARED UNDER OUR SUPERVISION BY FRANCISCO LOPEZ-GUTIERREZ BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHYSICAL AND CHEMICAL ALTERATION OF *Distichlis spicata* L.
CELL WALLS WITH NaCl AND WATER STRESS

Cultured cells of the halophytic grass *Distichlis spicata* adapted to grow in 500 mM NaCl (-25 bar) exhibited an altered growth physiology that resulted in slightly slower cell expansion and fully expanded cells with volumes only one-half to one-third those of unadapted cells. The reduced cell volume occurred despite maintenance of turgor pressures sometimes 2-fold higher than those of unadapted cells.

Tensile strength as measured by a nitrogen gas decompression technique showed empirically that walls of NaCl-adapted cells were weaker than those of unadapted cells. Correlated with this weakening was a substantial change in the organization of the matrix polysaccharides; specifically, glucuronoarabinoxylan was more readily extractable, perhaps caused by a decrease in cross-linkages with phenol substances. Despite a 3-fold decrease in the amount of hydroxyproline and approximately a 2-fold increase in tyrosine, the quantity of insoluble protein and, the proportions of crystalline cellulose remained relative constant. Glycosidic linkage analysis of carbohydrates revealed little modification in the quantity and primary structure of the matrix polysaccharides as a result of NaCl (salinity) and polyethylene glycol 8000 (water deficit) stress. The study

revealed that changes in the superstructure of glucuronoarabinoxylan are the primary determinant in the tensile strength of NaCl-adapted cells.

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INTRODUCTION

An understanding of the complexities of primary cell walls and their participation in environmental stresses is just emerging. Recent research concludes that: (a) the mechanisms that confer salt and drought tolerance to cultured cells and the molecular strategies by which cells are able to survive are not completely understood, (b) extensive studies have been focused on how salt and drought stress may alter the symplasm while the cell wall has been largely ignored and, (c) although there have been advances in knowledge of the chemical structure and synthesis of the primary cell wall, there is not a reliable model of it, as yet.

Primary cell walls play crucial roles in morphogenesis, disease-resistance, recognition, and signalling, as well as plant growth (Fry, 1988). Cell wall metabolism is an important component in plant growth, not only because cell walls comprise a large proportion of the cell biomass, but also because of the role of cell wall metabolism in determining wall extensibility (inverse viscosity) (Ray *et al*., 1972) for cell enlargement (Zhong and Lauchli, 1988).

Ordin (1960) reported that either NaCl or KCl, at 0.025 M, increased turgor pressure, cell wall synthesis, and cell enlargement in *Avena* coleoptile sections incubated in media containing mannitol. The salts increased turgor and slightly enhanced wall synthesis even at 0.175 M. Both NaCl and KCl enhanced cell

enlargement in cucumber hypocotyl segments (Purves, 1966) and in radish cotyledons (Nieman and Poulsen, 1967) as well. Ross (1974) suggested that light, cytokinins, wounding and a supply of osmotically active solutes; particularly, monovalent salts such as KCl, NaCl, or KNO_3, are important factors in the expansion of detached cucumber cotyledons. In fact, Ross and Rayle (1982) and Zack and Loy (1984) reported examples of cytokinin-induced growth in cucumber cotyledons and watermelon hypocotyls, respectively. It seems likely that cells of these tissues absorbed the salts, enabling them to absorb water osmotically and enlarge more. Obviously, they had adequate supplies of the metabolites needed for salt uptake and cell wall synthesis. They may differ in this respect from cells of intact salt-stressed plants.

Salt and drought stress are characterized by an inhibition of growth in most of the flowering plants (Flowers *et al.*, 1977; Greenway and Munns, 1980). This inhibition may be due to osmotic effects, ion toxicity, ion imbalance or a combination of these factors. Numerous and extensive studies have been devoted to understanding how salt and drought stress disturbs cellular processes within the symplasm, and how plants may cope with these conditions by means of osmoregulation, exclusion of salts, and sequestration of salts away from the cytoplasm. However, little information is available (Oertli, 1968; Binzel *et al.*, 1985; Flowers and Yeo, 1986; Moon *et al.*, 1986; Iraki *et al.*, 1989a, 1989b; Singh *et al.*, 1989) on what effects salt stress may have on processes in the apoplasm, such as cell wall metabolism and associated cell growth. Binzel *et al.* (1985) reported that wall "stiffening" might be a primary determinant in the inhibition of

cell expansion under osmotic stress. Iraki *et al.* (1989a; 1989b; 1989c) found significant changes in the composition of the wall matrix polysaccharides of saline-adapted tobacco cell lines. They also demonstrated that the cell wall tensile strength, as measured by the gas decompression technique (Carpita 1985), of adapted cells was much weaker than those of unadapted cells. These results indicated that a cellulosic-extensin framework is a primary determinant of absolute wall tensile strength, but complete formation of this framework apparently is sacrificed to divert carbon to substances needed for osmotic adjustment. One of the conclusions of Iraki *et al.* (1989a) was that the extent of this framework clearly is not a principal determinant of wall extensibility. The approach of using glycophyte- derived cell lines which have been adapted to tolerate salt and drought in long term tissue cultures appears to be promising for the understanding of cell wall metabolism under low external water potentials. An alternative to studying the adapted glycophyte cells is to use cell suspension cultures of halophytic higher plants which have significant cellular tolerance to salt. This should illustrate how cell wall organizational changes occur with salt tolerance mechanisms.

The goal of this research is to provide information on: (a) rates of change in cell volume, (b) changes of wall tensile strength and, (c) alterations of wall constituents of cell suspension cultures of the halophytic grass, *Distichlis spicata* (L.). Cultures were subjected to low external water potentials imposed by NaCl or polyethylene glycol.

LITERATURE REVIEW

Definition of terms

1) The physics of growth:

Plant growth Although some controversy exists between authors, plant growth can be defined as an irreversible cell wall extension and water absorption to increase the cell volume (Lockhart, 1965; Cleland, 1986). Plant growth is usually divided into cell enlargement and cell division; however, in both cases growth usually involves an increase in cell volume (i.e., dV/dt) and an extension of the wall already present (Cleland, 1986).

The water potential equation The water potential equation defines the net movement of water across a differentially permeable membrane (Salisbury and Ross, 1985) as:

$$\psi = \psi_{\pi} + \psi_{p} \qquad \text{Eq. 1.}$$

ψ = water potential (usually negative).

ψ_{π} = osmotic potential (negative), a property of the cell solution

ψ_{p} = turgor pressure or pressure potential (usually positive)

Growth rate Growth rate here refers to an absolute quantity (i.e., change in volume per unit time) according to Lockhart (1965) and Cleland (1987) as:

$$\frac{dV}{dt} = m(P-Y) \qquad \text{Eq. 2.}$$

In equation 2, V is the cell volume; thus (dV)/dt is the growth rate of the cell (dV = an incremental change in volume or cell size; dt = an infinitesimal increment of time). The *m* is the longitudinal wall extensibility ($bar^{-1}\ s^{-1}$), *P* is the turgor pressure and *Y* is the wall yield threshold (the critical pressure above which turgor-driven growth will occur) expressed in pressure units.

Elastic and viscoelastic extension When cell walls are subject to a constant stress (F/A= $dynes/cm^2$ or bars) they show three types of extension; elastic, plastic and viscoelastic (Cleland, 1987). In viscoelastic extension, the extension rate decreases with time (at a constant stress), but never falls to zero. Upon removal of stress there is a time-dependent contraction. In elastic extension the extension is instantaneous but remains constant with time until the stress is removed, whereupon the extension is completely reversed. In plastic flow, extension is directly proportional to time. When the stress is removed the extension undergoes no reversal (Cleland, 1986; 1987).

Wall loosening Wall loosening denotes the breakage of load-bearing bonds in the wall (Cosgrove, 1987b). Several studies have correlated auxin-induced wall loosening with hydrolysis of hemicellulosic polymers in the primary wall (Loescher and Nevins, 1972; Sakurai and Masuda, 1977; Yamamoto, *et al.*, 1980). However, the changes in wall chemistry that underlie wall loosening are controversial (Fry, 1989).

Wall "tightening" Wall tightening refers to the *de novo* deposition of wall material or the making of interpolymer cross-links as a response to an external stimulus (Fry, 1988).

Wall yielding Wall yielding refers to the irreversible extension or shearing of plastic elements in the wall (Cosgrove, 1987b).

Wall creep Wall creep denotes the physical change in wall dimensions at constant wall stress (Cosgrove, 1987b).

Wall relaxation Wall relaxation indicates the reduction in wall stress at constant cell wall dimensions (Cosgrove, 1987b).

The stress-bearing area of the wall In 1965, Roelofsen (cited by Taiz, 1984) proposed that the transverse stress is borne primarily by the inner wall layers containing transverse microfibrils, while the longitudinal stress is borne by the outer layer where the longitudinal microfibrils are located.

Tensile strength of the primary cell wall Tensile strength refers to the ability to withstand the tangential force per unit wall thickness resulting from the cell's internal pressure (Carpita, 1985). Tensile strength of primary cell walls can be estimated empirically either for spherical (eq. 3) or cylindrical (eq. 4) cells as:

$$\sigma_E = \frac{P \cdot r}{2t} \qquad \text{Eq. 3}$$

where σ_E is the equatorial wall stress, *r* is radius of the sphere, *P* is the hydrostatic pressure and *t* is thickness of the wall.

Since for cylindrical cells the stresses differ directionally the tangential stress (σ_T), which limits radial expansion of the cylinder, is about twice the longitudinal stress (σ_L equal to σ_E), which limits elongation (Nobel, 1983; Carpita, 1985; Taiz, 1984), thus the equation expands to:

$$\text{(a)}\ \sigma_T = \frac{P \cdot r}{t} \qquad \text{(b)}\ \sigma_L = \frac{P \cdot r}{2t} \qquad \text{Eq. 4}$$

Where σ_T is tangential stress, σ_L is longitudinal stress, with *P*, *r* and *t* defined as in eq. 3 (see Figure 12).

2) Description of the plant cell wall and its biological function:

The Cell wall A cell wall is a layer of structural material external to the protoplast. It is usually 0.1 to 10 μm thick and composed of polysaccharides, with smaller amounts of proteins, glycoproteins and phenolic compounds (Fry, 1988; Carpita, 1987; Bacic *et al.*, 1988).

Inorganic compounds are ubiquitous components of plant walls. Among them is silicon in the form of amorphous silica gel (opaline silica) (SiO_2 nH_2O) (Salisbury and Ross, 1985; Bacic *et al.*, 1988). Other elements, particularly metal cations such as Ca^{++} (Demarty *et al.*, 1984), are also found complexed to carboxyl groups on rhamnogalacturonans, glucuronoxylan, carboxyl or hydroxyl groups on phenolic acids and lignin monomers.

Primary and secondary walls Primary and secondary walls differ in chemical composition. This is not surprising in view of different roles ascribed to the two structures (Fry, 1988).

A typical primary cell wall is about 0.1 μm thick. In spite of this apparent thinness, it has to resist bursting in the face of a turgor pressure of several atmospheres (Carpita, 1985), but at the same time it must be plastic enough to allow an appropriate extent of growth. This occurs because the primary wall is a biphasic structure, consisting of relative rigid cellulosic microfibrils embedded

in a gel-like matrix composed of non-cellulosic polysaccharides and glycoproteins (Carpita, 1987; Fry, 1988).

Secondary walls Usually, higher amounts of lignin polymers are the distinctive characteristic of secondary walls. Hence, secondary walls may take on any of the wide variety of other important biological roles including defence, support and storage.

3) Sugar nomenclature and glycosidic linkage analysis:

Glycosyl residues A sugar residue glycosidically linked through its reducing carbon (C-1) is referred to as a glycosyl residue (Darvill *et al.*, 1980). For example, 4-linked glycosyl residues are glucosyl residues glucosidically linked at C-1 and which also have another glycosyl residue attached to them.

Glycoses Sugars with their reducing carbons free, whether or not the sugars have other glycosyl residues attached to them, are called glycoses, i.e., 4-linked glucose indicates a glucose that is located at the reducing end of an oligo- or polysaccharide and which has another glycosyl residue attached to it at C-4 (McNeil *et al.*, 1984).

Linkage analysis Linkage analysis can be referred to as the process of determining the glycosidic linkage of carbohydrates of the primary cell wall, deduced by gas chromatography-mass spectrometry (GC-MS) of partially methylated alditol acetates, and denotes carbon(s) participating in linkages. For instance, 4-linked xylosyl units represent xylosyl residues with C-1 (understood) and C-4 participation, whereas the actual derivative is 1,4-5-tri-*O*-acetyl-(1-

deuterio)-2,3-di-*O*-methyl pentitol; terminal (t-) arabinosyl units denotes non-reducing terminal arabinosyl units (Carpita and Shea, 1989).

4) Water deficit and salinity:

Water deficit For the purpose of this investigation, water stress or water deficit generally refers to a lowering of extracellular water activity, or water potential in the culture medium, by adding increasing amounts of polyethylene glycol-8000 (PEG). PEG is a polymer of ethane 1:2 diol (Yeo and Flowers, 1984).

Salinity Soil salinity is caused by an accumulation of soluble salts (Black, 1957; Bernstein, 1975).

Most saline soils are found in arid regions, although some saline soils occur in humid coastal areas as well. The major ions in saline soils are Na^{+}, Ca^{++}, Cl^{-}, Mg^{++}, SO_2^{-2}, and CO_3^{-2}. Minor ions include K^{+}, NO_3^{-} and $B_4O_7^{-2}$ (Black, 1957). Sodic or alkaline soils contain a high percentage of exchangeable Na^{+} (Haywood and Wadleigh, 1949). Some alkaline soils are saline, and some are not. Salinity is not to be confused with sodicity. Saline soils are not deficient in Ca^{++}; nonsaline-sodic soils are Ca^{++} deficient (Maas and Nieman, 1978). Black (1957) classified saline and alkali soils into four categories. These are saline soils, saline alkali soils, nonsaline alkali (sodic soils) and normal soils.

In this investigation a saline condition was created by adding increments of 50 mM NaCl (a highly soluble monovalent salt) into the culture medium.

Halophytes and their environment

Halophytes are plants that survive high levels of electrolytes in their environments. These environments are normally dominated by NaCl but may contain a variety of other salts such as Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl and Na_2CO_3 (Flowers *et al*., 1977; Waisel, 1972).

Epstein and Norlyn (1977) pointed out that there is not a fundamental incompatibility between plant life and highly saline environment, as evidenced by the existence of both marine algae and terrestrial halophytes. In fact, halophytes colonizing saline environments are from a variety of plant families suggesting that the evolution of salt tolerance is not restricted to a few families, and that tolerance has evolved independently in different taxa (Flowers *et al*., 1977). Jefferies and Rudmik (1984) reported that many halophytes, however, are members of three families: namely, the Chenopodiaceae, Gramineae, and Compositae. These are families containing many crop species. But the extent of the distribution of salt tolerance among members of the plant kingdom indicates there are limits to the process of evolution of tolerance. For example, tolerance to seawater has not evolved in families that contain important crop plants, including the Roseaceae and Rutaceae.

Actually, the survival of plants in any ecosystem depends on their physiological reactions to various stresses of the environment. Salt-sensitive plants, such as avocado, tend to slow their growth in response to 20 to 50 mM NaCl, whereas growth of desert halophytes and some mangroves is insensitive to 500 to 700 mM NaCl (Greenway and Munns, 1980).

Distribution of *Distichlis spicata* with respect to salinity

The halophytic graminoid spike grass, *D. spicata*, is a perennial grass common in the Atlantic coast tidal areas as well as inland saline marshes of many regions of North America including Mexico (Chapman, 1974; Hansen *et al.*, 1976). This species shows a very broad distribution with respect to soil salinity; it competes effectively in highly saline pannes (500-600 mM NaCl), as well as in areas of fresh water seepage (0-170 mM). However, it is most commonly found in areas of moderate soil salinity (250-425 mM) (Taylor, 1939; Ungar, 1966; Niering and Warren, 1974, 1980; Hansen *et al.*, 1976).

In laboratory growth studies of *D. spicata* from inland marshes, Hansen *et al.* (1976) found greatest growth at soil salinities of about 250 mM. However, Adams (1963) observed that optimal NaCl levels for greenhouse-grown *D. spicata* from North Carolina marshes was about 170 mM. Kemp and Cunningham (1981) also reported that growth rates of *D. spicata* under high light intensity in controlled environment chambers were reduced significantly by hydroponic solutions containing up to 500 mM NaCl. Apparently, from these studies and from field observations (Niering and Warren, 1980), *D. spicata* is likely to be one of the most tolerant grasses in the Atlantic tidal marsh community and may well require some salt for optimal growth (Hansen *et al.*, 1976).

Importance of halophytic grasses

Salt grasses are important pioneer plants in the early stages of succession. In salt marshes of southern Utah, salt grass contributes to a hammock-like

process that favors localized removal of salts by capillary action and evaporation (Hansen *et al.*, 1976). Because salt grasses are rhizomatous perennial they serve to stabilize the soil and reduce erosion in salt marshes and coastal sands.

Halophytic grasses have also been used as food and forage crops. The Seri Indians of the Sonoran Desert were known to harvest seeds of the grass *Distichlis palmeri* on the delta of the Colorado River (Felger, 1979). The use of silage of *Spartina*, a genus of halophytic grasses, has been reported in the U.S. (Kirby and Gosselink, 1976) and in Great Britain (Hubbard and Ranwell, 1966). In the dry lake of Texcoco, near Mexico City, native *D. spicata* grows in saline soils and serves as the total dietary forage for cattle (Urbina, 1980; cited by Gallagher, 1985).

The ability of *Leptochloa fusca* (kallar grass) to grow in salt-affected soils in Pakistan, and produce biomass from which pulp, compost biogas, and fuel alcohol could be obtained (Pakistan atomic energy commission, 1987), constitutes a clear example of the potential of halophytes in the development of marginal lands. Most importantly, they eventually may serve as a source of genes for improving the salt tolerance of crop species (Epstein and Norlyn, 1977; Gallagher, 1985; Tal, 1985).

D. spicata and the closely related *D. stricta* are both relatively small plants, mostly less than about 30 cm in height (Somers *et al.*, 1979). A yield of dry matter of 6,600 kg/ha has been reported for the former (Mudie, 1974). It is grazed by horses and cattle along the shores of the Gulf of California (Mexico) near the Colorado River delta. The seeds contain approximately 14% protein and

are small (average about 0.8 mg each) but are frequently produced in abundance.

Some responses of halophytes to salinity

A typical response of many plants to saline environments, particularly halophytes, is to accumulate high intracellular concentrations of Na^+ and Cl^- (Flowers *et al*., 1977; Greenway and Munns, 1980; Wyn Jones, 1981; Yeo, 1981; Storey *et al*., 1983). Halophytes, in general, are unique in their ability to accumulate concentrations of salts equalling or exceeding those of sea water in their leaves without injury. Furthermore, Na^+ accumulation has a positive function in dicotyledonous halophytes. Such species are normally faced with maintaining a high cell water content in the presence of a low external water potential caused by the high salinity. Therefore, the ion transport system maintains a source of ions for osmotic adjustment, absorbs nutrient ions when they are unusually outnumbered by the saline ions, and distributes these ions in a manner consistent with the requirements of the plants' metabolic processes.

A vital feature of the physiology of halophytes is their ability to control salt uptake and compartmentalization, especially in leaves, to maintain turgor for growth while avoiding either a water deficit or an excess of ions, either of which could prove toxic.

The effects of salinity on the accumulation of ions by halophytes has been summarized on numerous occasions (Flowers *et al*., 1977; Flowers *et al*., 1986). For example, in the dicotyledonous halophytes these ions can amount to 30 to

50 percent of the dry weight, and are predominantly Na^+ and Cl^-, generating $Na^+:K^+$ ratios in the plant often in excess of 10 (Flowers *et al.*, 1986). However, not all species rely on Na^+ to adjust their osmotic potential. Many monocotyledonous halophytes have $Na^+:K^+$ ratios of about one or less. It appears from the data in the literature that the major separation between monocots and dicots, with regard to halophytic behavior, is that the former have much lower $Na^+:K^+$ ratios than the latter and in general much lower water contents (Flowers *et al.*, 1977; Gorham *et al.*, 1980; Flowers *et al.*, 1986).

Therefore, some of the characteristics of halophytes that may suggest some physiological mechanisms involved in salt tolerance are: (1) succulence that may lead to dilution of intracellular salt, for example in *Salicornia* spp., (2) the presence of salt excreting glands, particularly in the leaves, that reduce the levels of salt in the plant (Waisel, 1972), (3) development of small leaves, water storage hairs and aerenchyma, (4) a water potential range from -2 to -4 MPa, which is equivalent to an ion concentration of 400-700 mM, (5) synthesis and accumulation in the cytoplasm of proline, glycinebetaine and sugars, and the ability to compartmentalize Na^+ and Cl^- in the vacuole as well. (6) induction of crassulacean acid metabolism (CAM) photosynthesis, for example in the ice plant *Mesembryanthemum crystallinium*, and other salt-tolerant succulents (Winter *et al.*, 1981).

Hansen *et al.* (1976) and Oross and Thompson (1982) reported that the leaf epidermis of *D. spicata* contained salt glands that were active in the extrusion of salt. Kemp and Cunningham (1981) observed that increased salinity resulted

in generally thicker leaves with lower stomatal density but no significant differences in the ratio of mesophyll surface area to leaf area. In addition, salinity and light during growth did not significantly affect rates of dark respiration. These authors concluded that the mechanisms by which *D. spicata* tolerates salt appear to be closely coupled to the use of light energy. On the other hand, Smart and Barko (1980) demonstrated that in addition to salt exclusion, the roots of *D. spicata* and *Spartina alterniflora* also function effectively in ion selection. A selective uptake of K^+ and exclusion of Na^+ occurred in the roots of both grasses. These processes increased the Na^+:K^+ ratio in the interstitial waters and also resulted in increased sediment salinities.

It is clear, therefore, that salt tolerance in *D. spicata* is the result of several processes.

Use of cell cultures *in vitro* to study salt tolerance

In recent years cells of halophytic plants have been grown *in vitro* in the presence of various levels of NaCl. Resistance has been assessed in different ways, such as growth, survival and metabolic status. No direct correlation has been found between *in vitro* performance of cells and the *in vivo* growth of plants (Smith and McComb, 1981; Flowers *et al*., 1985; Ochoa-Alejo and Lopez-Gutierrez, 1987). In some cases, however, callus and cell suspension cultures paralleled the plant response to salt (Tal *et al*., 1978; Orton, 1980; Warren and Gould, 1982). Cell cultures allow the processes or physiological markers involved in salt resistance to be characterized at the cellular level. The relative lack of

differentiation in the cultured cells eliminates complications arising from the morphological variability of differentiated cells of the various tissues of whole plants Staverek and Rains, 1983).

The use of in vitro cultures offers advantages in studies of plant cell responses to water stress as well. According to Hasegawa *et al*. (1984), a cell culture system allows the elimination of all the water stress responses, except those that are operative at the cellular level. Measurements of water relation parameters (ψ, ψ_{π}, ψ_{p}) representative of the whole population can be made easily on a small sample of cells. Furthermore, the cultured cell population is composed of rather uniformly growing cells, hence, physiological and biochemical changes in response to water and salt stress represent those associated with growing cells.

The water potential of the incubation medium may be lowered to simulate water deficits by using polyethylene glycol (PEG), M.W. 6000-8000 (Bressan *et al*., 1982; Handa *et al*., 1986; Rhodes *et al*., 1986). Heyser and Nabors (1981) reported on the use of PEG 4000 and dextran as nonpenetrating osmotic solutes to lower the medium water potential for tobacco cells. Sucrose and mannitol have also been used as osmotic solutes (Gollek, 1973; Thimann *et al*., 1960). In general, an ideal osmoticum should have two properties. First, it should be inert so that it does not affect the metabolism of the cells other than as a solute that lowers the osmotic potential. Second, for many studies, the ideal osmoticum should be nonpenetrating. Only nonpenetrating solutes do not introduce errors in the determination of water potential by gravimetric methods (Slatyer, 1967).

It appears that PEG has partially replaced sucrose and mannitol as an experimental osmoticum. As is common for many large MW compounds, PEG does not follow van't Hoff's equation (Applegate, 1960; Money,1989). At higher PEG concentrations the measured osmotic potential is considerably greater than that predicted by the van't Hoff equation (Applegate, 1960). Thus, the actual osmotic potential produced by a given PEG concentration should be experimentally determined, as Bressan *et al.* (1982) and Money (1989) have done and many others before them.

Restricted plant cell expansion caused by salt and drought stress

The mechanisms that confer salt and drought tolerance to cultured plant cells and the molecular strategies by which cells are able to survive in these environments are not completely understood. In recent years, the process of osmotic adjustment in higher plants has been studied extensively (Turner and Jones, 1980; Hanson and Hitz, 1982; Kramer, 1983;). From these studies, it is clear that a cellular osmotic potential has to become more negative as a requisite for growth and survival when plants are exposed to salt and drought conditions; otherwise, cells would lose turgor to a point restricting cell expansion (growth). Salgado-Garciglia *et al.* (1985) reported a 38% cell size reduction (volume) in sweet potato cells adapted to 1% NaCl; the reduction in size was partially reversed when the cells were transferred to a salt-free medium. Binzel *et al.* (1985) found that tobacco cell lines adapted to 1.0% to 3.5% NaCl showed a cell volume reduction according to NaCl concentration. This reduced cell volume

associated with adaptation to salt stress occurred despite maintenance of a more than adequate turgor needed to drive cell expansion under normal conditions. Consequently, a reduction of wall extensibility, i.e. a wall "stiffening", was proposed as a reason for the occurrence of small tobacco cells in lines adapted to saline stress.

Meyer and Boyer (1981) suggested that a reduced growth rate may actually be a part of the osmotic adjustment process, because they observed a reduction of growth without loss of turgor in the growing tissues of soybean seedlings under desiccation stress. In growing regions of barley leaves, Matsuda and Riazi (1981) also found a restricted growth rate without loss of turgor. Similar observations of decreased growth despite turgor maintenance have been made with tissues subjected to reduced external water potential (Cutler *et al.*, 1980; Hsiao *et al.*, 1976; Matsuda and Riazi, 1981; Michelena and Boyer, 1982). Based on these observations, it has been acknowledged that in walled cells osmotic adjustment is paralleled by a subtle cell wall elastic adjustment which in turn, determines at least in part, cell expansion (Hsiao *et al.*, 1976; Van Volkenburgh and Boyer, 1985; Binzel *et al.*, 1985; Bolanos and Longstreth, 1984).

Principles of plant cell expansion

The shape of plant cells is dictated by the cell wall, a meshwork of polysaccharides and structural proteins surrounding each cell. Growth and differentiation in plants is manifested in the coordinated expansion of the existing cell wall matrix and the assembly of newly synthesized polymers onto cellulose

microfibrils. These processes in grasses are further specialized because the chemical structure of the expanding cell walls is so different from those of dicot and other monocot cells (Bacic *et al.*, 1988; Carpita, 1987).

According to Ray (1987), the permanent growth of a plant cell requires that its cell wall undergo a permanent expansion. This is called an irreversible extension, to distinguish it from the reversible (elastic) extension that must exist in the wall of any cell when the cell possesses a turgor pressure. In addition, Ray (1987) stated that a cell may grow throughout its surface, or throughout its length in the case of a cell that is elongating. This is termed diffusive growth; irreversible expansion is occurring in all parts of the cell's wall, or in its side walls in the case of a diffusely elongating cell. Some types of cells instead show localized growth resulting from irreversible extension occurring only in a local area of the cell wall. The most common instance is tip growth in which a cell elongates into a tubular shape by wall expansion restricted entirely to the tip of the tube; for example, root hairs, pollen tubes, and fungal hyphea.

Some aspects of cell wall mechanical properties

As noted above, a tip-growing cell can achieve an elongated shape by restricting wall loosening to a localized patch at the tip of the cell. Wall loosening refers to a cell's capacity for its wall to undergo irreversible extension presumably by the breakage of load-bearing bonds (Cosgrove, 1987b; Ray, 1987).

Taiz (1984) pointed out that primary walls must be extremely strong to resist the tendency of the protoplast to absorb water in response to a large osmotic gradient. However, what is remarkable about primary walls is not their rigidity, but their ability to undergo metabolically controlled extension in response to turgor pressure, allowing plant cells to enlarge by an order of one magnitude or more.

Lockhart (1965) first recognized that cell enlargement can be described by the equation:

$$dV/dt = m(P-Y)$$

where the rate of cell elongation (dV/dt) is the product of the wall extensibility *(m)* and the difference between turgor pressure *(P)* and the wall yield pressure *(Y)*. From Lockhart's equation, it can be deduced that the ability of a plant cell to enlarge depends, in part, on the extensibility of the cell wall.

Any change in m will result in change in the growth rate, as long as P and Y remain constant. A more explicit definition for Y is given by Cosgrove (1985) as follows: Y is the critical turgor for growth (the yield treshhold). Therefore, it appears that to understand how cell expansion is regulated, it is necessary to understand what m represents and how it can be controlled. To do this, Cleland, (1987) suggested that four questions must be asked. First, what are the intrinsic extensibility properties of plant cell walls? Second, are they identical to, or different from, the characteristics of m as deduced from the properties of *in vivo* cell extension? Third, is it possible to obtain *in vitro* wall extension with

characteristics identical to those of *in vivo* extension? And, finally, how does wall loosening (e. g., an increase in *m*) occur *in vivo*?

The primary cell wall is a polymeric substance; hence, it will have a set of extensibility properties. For instance, the mechanical properties of the walls are always different quantitatively, and possibly qualitatively, when subjected to unidirectional stress (applied force) as compared with a multidirectional stress (turgor). With applied force the extension can be up to 3-times greater than with the same longitudinal stress produced by turgor. Kamiya *et al*. (1963) were able to determine this relationship experimentally with *Nitella* cells.

Cleland (1984) pointed out another problem concerning the conditioning of the walls and the direction of the force vectors. Briefly, when an elastic material is extended twice to the same length, with the same force vectors, the extension characteristics of the second extension (conditioned) are different from those of the first extension (unconditioned). Hence, a cell wall *in vivo* may act as a conditioned or an unconditioned material, depending upon its past history of turgor. The walls of a cell that has experienced no history of reduced turgor will probably appear to be unconditioned, but the walls of a cell that has shrunk slightly due to a loss of turgor would be expected to act as a conditioned material. Most studies on the extensibility of polymeric substances have been restricted to conditioned material, while almost all studies on cell walls have been confined to the unconditioned state (Cleland, 1987).

Ray (1967) and Kutschera (1987) have reported differences in thickness between the outer epidermal walls (OEW) and inner tissue walls (ITW) of maize

coleoptiles. A comparison of the thickness of OEW versus ITW in IAA-sensitive plant organs reveal that OEW is about 5 times as thick as walls of the inner tissues. In pea epicotyls (Lang *et al.*, 1982) the rigid OEW shows a crossed polylamellate structure in which lamellae having a longitudinal orientation of cellulose microfibrils alternate with lamellae of mainly transverse orientation. The microfibrillar orientation of the ITW, on the other hand, seems to be predominantly transverse. These structural differences in wall thickness and architecture of microfibrils may affect the extensibility properties and the biochemical capacities of tissues in an organ. Thimann amd *scjmeoder (1938) reported that the two halves of longitudinally split stems and coleoptiles bow outward when floated in water, suggesting that the central tissues were normally under compression while the epidermis was under longitudinal tension.

In general, three main techniques have been employed for measuring the intrinsic extensibility (*m*) of the cell wall: creep, stress relaxation and Instron measurements (Cleland, 1986). In a creep test (Cleland, 1971), the walls are subjected to a constant applied force and the extension is measured as a function of time. In stress relaxation (Masuda, 1978), the walls are rapidly extended, then held at a fixed length, and the decay in stress across the walls is measured as a function of time. In the Instron assay (Cleland, 1967), a tissue is extended at a constant rate of extension, and the stress along the walls is measured as a function of the amount of extension.

Of these, creep (constant load) most closely resembles *in vivo* extension (Taiz, 1984). It is worth noting that for the above techniques higher plant cell wall

preparations traditionally have been boiled in methanol and then rehydrated to eliminate the undefined effects of enzymes. Hence, such preparations behave as purely physical systems with a Q_{10} (temperature coefficient) close to 1 for extension (Cleland, 1971).

A new technique has recently been devised to permit *in vivo* stress relaxation (Cosgrove, 1985). The pressure-block technique involves cutting the supply of water to a growing tissue, resulting in turgor relaxation or change in balance (or block) pressure which is monitored. Kinetic analysis to calculate *m* requires knowledge of ϵ (volumetric elastic modules), and the total process requires 2 h or more to obtain a single value of *m*.

The cell wall elastic and viscoelastic characteristics are beginning to be revealed. According to Cleland (1987), when cell walls are subjected to unidirectional stress (applied force) *in vitro*, the extension is dependent upon the wall loosening events which are basically bond breakage, chemical creep and wall synthesis. But measurements of extension may vary because of the mechanisms involved. In walls of *Avena* coleoptiles, the extension is in response to acidic pH and appears to involve the enzymatic cleavage of some glycosidic bonds. In soybean walls, the extension occurs in response to either the removal of wall Ca^{++} crosslinks or to low pH and has a non-enzymatic component (Virk and Cleland, 1986). Still, the mechanisms of wall extension remain unknown in growing tissues (Cosgrove, 1987b).

The physical theory of plant cell growth suggests that the rate of cell expansion is determined by the rate of two simultaneous physical processes:

water uptake and irreversible cell wall expansion. Both are needed for growth; however, which comes first?

Recently, Cosgrove *et al.*, (1984), Cosgrove (1985; 1987a) and Van Volkenburgh and Cleland (1986) have strongly suggested that growth starts with a subtle biochemical modification, or rearrangement of the cell wall. Because a reduction or relaxation of wall stress is the reactionary force to turgor pressure, stress relaxation of the wall lowers cell wall turgor pressure and water potential and thereby induces a water influx by osmosis. Hence, it seems likely that the changes in mechanical properties of the wall that occur during and at the termination of expansion are under metabolic control.

Tensile strength of the primary cell wall

The mechanical strength of some plant organs is caused by the turgidity of component parenchyma cells. These cells make up the pith, all or most of the cortex of roots and shoots, and mesophyll of leaves. Their walls are usually thin and unlignified, but nevertheless have sufficient tensile strength to resist high turgor pressures (Bacic *et al.*, 1988). In contrast, walls in other plant organs are the load bearing elements. Thick, lignified walls, such as those of tracheids and fibers, provide mechanical support and rigidity to many structures, for instance the trunks of trees and grass stems. Although unlignified walls can withstand tensile forces, lignified walls are better able to withstand compressive forces that are imposed on aerial parts by gravity and winds (Raven, 1977). Silification of walls is probably another strategy for mechanical support, particularly in grasses,

since silica accumulates in the walls of most of the cells of these species including epidermal cells walls (Salisbury and Ross, 1985). Although it is acknowledged that walls give tensile strength to maintain turgor, little is known about the tensile strength of walls of single cells because of technical problems in making such measurements mechanically (Carpita, 1985). However, Carpita (1985) devised a gas decompression technique to determine empirically the tensile strength of singled carrot cells. Tensile strength must be calculated from pressure, cell diameter, and cell wall thickness, and because the force applied by the large cell volume is resisted by an extremely thin wall, tensions developed within the wall are enormous compared to the turgor pressures (Carpita, personal communication). There are few examples in recent literature where the tensile strength has been estimated (Carpita, 1985; Iraki *et al.*, 1989a). This aspect, therefore, is open for more research.

Some features of primary cell wall chemistry

The primary cell wall is a vital organelle with unusual physical and chemical properties. It is composed of polymers, some of whose structures (and therefore biosyntheses) are among the most complex known (McNeil *et al.*, 1984). The wall contains enzymes that are catalytically active *in vivo,* and the wall dramatically changes structure during development (Cassab and Varner, 1988). Indeed, the complex chemistry occurring in a dynamic extracellular matrix helps to rationalize the presence of cell wall enzymes. The predominant view now is that the peroxidase system tends to increase the number of cell wall cross-links,

or at least it is involved in lignin polymerization wheras glycosidases tend to decrease cross-linking (Fry, 1985; 1989).

Primary cell walls of members of the Poeaceae (formerly Gramineae), in particular cereal grasses, are remarkably different in chemical composition from those of the Dicotyledonae and other Monocotyledonae (Bacic *et al.*, 1988; Carpita, 1987). Further, Carpita (1984; 1987) has reported that walls of the cereal grasses synthesize different polymers during either cell division or cell expansion.

The chemical composition of cell walls from *Acer pseudoplatanus* and *Zea mays* are examples of the major differences between the primary walls of the cereal grasses and dicots (Table 1).

In general, the walls of dicots are viewed as a framework of cellulose microfibrils with xyloglucan principally, and with some arabinoxylans hydrogen-bonded to the surface of the microfibrils and spanning the milieu between them (Bacic *et al.*, 1988). This reinforced framework is then embedded in a matrix of polysaccharides, including polygalacturonan and rhamnogalacturonan (Bacic, *et al.*, 1988). The polyuronans comprising the gel are organized via Ca^{++} electrostatic interactions and phenolic ester and ether linkages (Fry, 1986).

Lamport and Northcote (1960) showed the first evidence that extensin was an integral part of the primary cell wall and which contained virtually all of the hydroxyproline of the cell. Extensin is a hydroxyproline-rich glycoprotein (HRGP) with intramolecular isodityrosine linkages that reinforce a "polyproline- II-like" helix into a stiff rod-like molecule (Strafstrom and Staehelin, 1986; Cooper *et al.*, 1987).

Table 1. Primary cell wall composition of dicots and Graminaceous monocots.

Component	*Acer pseudoplatanus* (a) Cell suspension cultures	*Zea mays* (b) coleoptile
	% wt. of cell walls	
Pectin:	34:	10:
(1→4)α-D-galacturonic acid	+ + +	+
rhamnogalacturonan I	+ +	+
rhamnogalacturonan II	+	?
arabinan, galactan, AGP	+ +	+
Hemicellulose:	24:	65:
xylogucan	+ + +	+
glucuronoarabinoxylan	+	+ + +
mixed-linked ß-D-glucan	?	+ +
Protein:	19:	10:
Extensin	+ +	+ /-
AGP	+ +	+ +
Cellulose	23:	15:

[a] Data from Keegstra *et al.* (1973). [b] Data from Carpita (1983) and Carpita and Kanabus (l988). The symbol (+) denotes proportion of wall constituents.

Through proposed intermolecular isodityrosine linkages, or yet unknown covalent linkages, the extensin network can form a "warp-weft" arrangement, enveloping the individual cellulose microfibrils and establishing a rigid matrix (Lamport and Epstein, 1983). The "warp-weft" interaction was suggested as a determinant of wall expansion during growth through reversible changes in the organization of the cellulose extensin network (Lamport and Epstein, 1983).

Likewise, the graminaceous monocot wall contains cellulose microfibrils but these are hydrogen bonded primarily with arabinoxylans and some xyloglucans, a portion of which are immobilized by phenolic ether cross-links (Carpita, 1987). Acidic arabinoxylan molecules are hydrogen bonded to each other and may span the matrix; additional glucuronoarabinoxylan molecules may define the pores of the matrix, replacing the function of pectic substances which are few in these species.

The highly substituted glucuronoarabinoxylan may be the nascent xylan and is cross-linked loosely through diferulic acid (Carpita, 1987). A mixed-linkage ß-D-glucan also comprises a substantial part of the hemicellulose of cereal grasses. Distinct from (1→3)ß-D-glucan, callose and (1→4)ß-D-glucans of other hemicellulosic polymers, this mixed-linkage polysaccharide is unique to grasses and is an unbranched polymer containing both (1→3)ß-D- and (1→4)ß-D-glucosyl units in a ratio of about 1:3 (Bacic *et al*., 1988; Carpita, 1987).

Although much is known about the sugar and amino acid sequences of the wall polymers (Darvill *et al*., 1980; McNeil *et al*., 1984; Bacic *et al*. 1988; Fry, 1988; Carpita, 1987 little is known of the cross-links that hold the wall matrix

together. Nevertheless, the models described above either for dicots or monocots are considered by some workers as a prototype for a noncovalent association between polymers (Carpita, 1987; Bacic *et al.*, 1988; Fry, 1986); in turn, these models might serve as the primary basis for wall integrity. Hence, H-bonds, Ca^{++}-bridges, other ionic bonds, coupled phenols and ester bonds all likely play a role in building the primary cell wall, although other cross-links may also be involved (Fry, 1986).

The model proposed by Keegstra *et al.* (1973) for the dicot primary wall depicted most of the matrix polymers (pectins, hemicelluloses) as covalently linked to one another. According to Ray (1987) this model, construed as a structure requiring covalent bond breakage for its extension, has heavily influenced thinking about wall loosening, even though considerable information regarding wall polymers is contrary to its representation of most of the wall's matrix polymers as being glycosidically covalently interlinked. Further, a covalent network has not been demonstrated yet in plant cell walls (Ray, 1987).

The prototype depicting the plant cell wall as a biphasic structure, consisting of a rigid skeleton of cellulose microfibrils held together by a gel-like matrix, has to be improved, for instance, by unveiling cross-linking of the matrix polymers.

Accurate three-dimensional views of the wall are needed to understand fully the physical constraints and the actual mechanisms of microfibril separation and wall expansion, but are not currently within reach (Carpita, 1987). Hence,

there are many unanswered questions on the relationship of the cell wall and growth.

A true appreciation for the dynamic nature of the cell wall has recently emerged. Advances in methodologies to study the chemical structure, metabolic processes, and mechanical properties of the primary cell wall, make it possible to formulate proposals for understanding the complex interaction between the primary cell wall and its aqueous environment.

OBJECTIVES OF THE DISSERTATION RESEARCH

The physical properties of wall expansion and control of growth rate through changes in cell turgor and wall extensibility are well substantiated (Taiz, 1984; Cleland, 1987), but the biochemical modifications responsible for these changes are not completely defined. Iraki *et al.* (1989a) reported that tobacco cells adapted to 428 mM NaCl accumulate dry weight at rates comparable to unadapted cells but exhibit as much as a 5-fold decrease in average cell volume and a reduced rate of cell expansion. This occurs despite an osmotic "overadjustment" that results in development of turgor pressures several fold higher than in unadapted cells. They found also an intriguing loss of absolute tensile strength in walls of adapted cells, that was correlated with a reduction in the mass of cellulose-extensin network. In companion studies, Iraki *et al.* (1989b; 1989c) suggested that a substantial change in the chemical composition and organization of the pectic substances in walls of adapted cells to water (PEG) and saline stress, indirectly may participate in reducing the ability of the walls to extend. The reduced levels of soluble pectins and xyloglucan in the medium of both NaCl- and PEG - adapted cells led them to hypothesize about an impaired turn-over of matrix polysaccharides. In turn, this impairment may account for the inhibition of growth resulting from adaptation to osmotic stress.

Based on the preceding observations, similar methodologies as those of Iraki *et al*. (1989a) were used to seek answers in a different model cell system: a halohytic cell suspension culture. Once the different cell lines were established; changes in cell volume (expansion), alteration of the wall tensile strength and modification of wall constituents associated with saline and water deficit stress were explored.

MATERIALS AND METHODS

Cell cultures

Suspension cultures of *Distichlis spicata* L. were kindly provided by Dr. Ray Ketchum, Tissue Cultures for Crops Project, Colorado State University. Cells were maintained in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium at 28°C$\pm$2°C and 120 rpm in continuous light. Every ten days a 10 mL aliquot of the cell suspension (1.0 to 1.7 g fresh weight) was used to inoculate 90 mL of fresh liquid medium containing major and minor salts and vitamins of Murashige and Skoog (1962) medium (MS) supplemented with 10^{-6} M 2,4-dichlorophenoxyacetic acid and 20 g/L sucrose, pH 5.75.

Cells were adapted to grow in a medium supplemented with either various concentrations of NaCl, up to 500 mM (by adding 50 mM with each passage) or 20% and 25% (w/v) polyethylene glycol 8000 (PEG). In order to adapt cells to grow in PEG, it was necessary to grow the cells in 10% PEG prior to moving them into 20 and 25% PEG. An initial concentration of 20% PEG prevented cell growth even at a density of 1 to 5×10^{6} cells/ml. Hence, lower PEG concentrations were used in each step of the adaptation. Cells adapted to grow in 400 and 500 mM NaCl as well as 20 and 25% PEG were subcultured into fresh medium containing the respective level of osmotica after 18 days of culture. The unadapted and 200 mM NaCl cultures were subcultured into the appropiate

medium after 10 days. Cell lines adapted to grow in these media were maintained for at least six months prior to use in the experiments described here.

Determination of cell number

Cell counting procedures were a modification of those of Warren and Gould (1982). One-mL aliquots was removed from the suspension cultures and mixed with 4.0 mL of either 10% or 15% (w/v) aqueous chromium trioxide. This mixture was incubated at 65°C for 20 min and then vigorously vortexed for 1 to 2 min.

Cells were counted under a microscope using an AO Bright-Line hemacytometer; a minimum of 256 fields were counted for each sample of adapted and nonadapted cell line used in the experiments.

Determination of fresh and dry weights

Cells were harvested on Whatman no.1 filter paper in a Buchner funnel with aspiration. Fresh weights were recorded and cells were allowed to dry at least 24 h in an oven at 80°C before determining dry weights.

Determination of cell volume during growth of cell suspension cultures

Cell volumes were estimated by determining the fresh weight minus dry weight of culture samples after collecting cells by vacuum filtration and dividing by the total number of cells in samples of equivalent weight (Binzel *et al*., 1988). Volume values obtained by this method also correlated well with empirical

measurements of cell length and width and calculation of volume using simple equations for spherical or ellipsoid solids (Binzel *et al.*, 1987; 1988). All experiments contained triplicate samples, and each experiment was done twice, except as noted. For example, each experiment consisted of 54 Erlenmeyer flasks of 50 mL containing 22.5 mL of MS liquid culture medium, supplemented with different levels of NaCl or PEG. Two and half mL (0.55 to 0.6 g fresh weight) of cell suspension were inoculated into each flask and incubated in a shaker (110 rpm) at room temperature for 18 d. After two days of culture, three replicates were harvested and volume changes were determined. This procedure was repeated throughout the growing cycle for each experiment. According to Iraki *et al.* (1989a) the method of Binzel *et al.* (1988) may result in an overestimate of cell volume because there is no correction for extracellular volume including cell free space. However, if care is taken in the filtration process, extracellular volumes should be relatively constant from sample to sample. Some differences in extracellullar volume between adapted and unadapted cells would be expected because of cell size differences. But this effect should be relatively constant as cell samples are taken over time.

Determination of cell size

One mL of cell suspensions from cultures in early stationary phase of growth were harvested and suspended in 1 mL of 10% (w/v) of chromium trioxide contained in a scintillation vial. This mixture was heated at 65°C for 10 min, then carefully mixed and cooled at room temperature. The length and width

of the cells were empirically determined using a light microscope with the aid of an hemacytometer and a calibration microscale. In all experiments at least 40 measurements for both length and width were made; the arithmetic mean was taken as the representative value for each parameter.

Determination of osmotic potential of concentrated liquid cultures of NaCl or PEG

Measurements of the osmotic pressure of the aqueous culture media were made according to the Wescor Instruction Manual (1984). All determinations were made on fresh liquid cultures at 21°C $\pm$ 1°C and after the cell lines reached their respective stationary growth phase.

Eight-μL samples of the culture medium were pipetted onto 6 mm diameter filter paper discs setting in the sample chamber of a Wescor 5100C Vapor Pressure Deficit Osmometer (Wescor, Inc., Logan, UT). The osmolality of each solution was measured repeatedly until a series of three readings was obtained that lay within a 5 mosmol range (minimum of three replicates per NaCl or PEG concentration). A mean value of osmolality was calculated from the closest three measurements. The osmometer was frequently recalibrated over the range of 100 to 1000 mmol/kg using NaCl osmolality standards (Wescor, Inc.).

Osmolality measurements were converted to π (bar) using the formula π = -miRT; where π = osmotic potential, m = molality of the solution, i = an ionization constant related to the solute, R = the gas constant (0.0831 litre bars/mol. K) and, T = absolute temperature (K). Sodium chloride and PEG-8000

(PEG 6000-8000 M_r) were purchased from Sigma Chemical Co., St. Louis, MO., USA.

Determination of turgor pressure of adapted and unadapted cells to high levels of NaCl

Turgor pressure of adapted and unadapted cells grown in media up to 500 mM NaCl were measured by determining the incipient plasmolysis in graded NaCl and sucrose solutions of 0.1 molal units and determining the water potential of the culture medium. The difference between these values is equivalent to the turgor pressure according to the equation: $\psi_p = \psi - \psi_\pi$

General theoretical and practical aspects of incipient plasmolysis determination have been described (Ross, 1974; Nobel, 1983). Osmotic potential procedures were a modification of those of Ross (1974) and Handa *et al*. (1982). Turgor pressure was calculated as the difference between water potential of the growth medium (determined by vapor pressure deficit osmometry) and osmotic potential causing incipient plasmolysis of 50% of the cell population. Cells were harvested from stationary growth phase cultures and centrifuged at low speed to form a loose pellet, and 40 μL of the cell suspension were added to 400 μL of each NaCl or sucrose solution containing neutral red dye (0.1%). After 30 min, the cells were observed by bright-field microscopy. Any visible separation of the plasmalemma from the wall was scored as plasmolysis. There was ample variation within the cell population regarding plasmolytic behavior; thus incipient

plasmolysis was defined as occurring when 50% of the cells were visibly plasmolyzed. At least 200 cells were counted in each experiment.

Determination of tensile strength of primary cell walls

Carpita (1985) established the theoretical and practical frame-work for the measurements of tensile strength of cell walls of singled cells. Experiments described here used unadapted and NaCl-adapted cells. Technical problems prevented determination of tensile strengths in cells in the highly viscous PEG medium. The procedures were slightly modified from those of Carpita (1985).

Fifteen mL of cell suspensions in stationary growth phase were collected and transferred into a scintillation vial. This vial was placed in a nitrogen gas decompression bomb (Parr Instruments) with gentle stirring to suspend the cells (Figure 13). Nitrogen gas was introduced slowly (to avoid heating) in the cell suspension through a side valve to the desired pressure (up to 150 bars). After equilibration for 15 min, the cell suspension was jettisoned to ambient pressure via a second valve and a tube that extended into the suspension. Thus, the cell suspension was collected into a large cylinder. An aliquot was mixed with one-half volume of 20% (w/v) chromium trioxide in a 3-mL Reacti-vial (Pierce) and stirred gently for 1 h for cell counting. If the pressure differential induced by the gas plus turgor pressure was greater than the breaking strength of the wall, the cell exploded. The number of cells that survived decompression was visualized by bright-field microscopy and counted with a hemacytometer.

The percentage of intact cells was plotted against the pressure differential, and breaking pressures above turgor were calculated as the pressure differential required to break 50% of the cell population.

Preparation of wall material

Cells were harvested at the early stationary phase (8 d) for the unadapted and adapted lines to 200 mM NaCl and 16 d for those adapted to grow either with 400 and 500 mM NaCl or 20 and 25% PEG. The cell suspensions were filtered by aspiration through Whatman no. 1 filter paper, and the cells were recovered, frozen and lyophilized.

Isolation of cell walls

Cell walls of cell suspensions were obtained using a procedure similar to that described for other tissues (Carpita, 1983). Two g of lyophilized cells were homogenized (0.5 g/10 mL each time) in a Duall (Kontes) glass-glass homogenizer with ice-cold 50 mM N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (Tes buffer) containing 10 mM ascorbate, pH 7.2, followed by centrifugation for 10 min at 3500 rpm. Ten mL of the supernatant of each sample were mixed with 40 mL of ice-cold absolute ethanol to make a 80% (v/v) solution with respect to ethanol. This precipitated proteins and soluble polysaccharides. The ethanol-insoluble material was frozen and lyophilized.

The insoluble material was sequentially washed and recovered by centrifugation as follows: four times with ice-cold 0.5 M potassium phosphate

buffer, pH 7.0; twice with deionized water; twice with chloroform-methanol (1:1 v/v) at 45°C for 30 min each; once with methanol; and twice with deionized water. Then the insoluble material was resuspended in 10 mL of dimethylsulfoxide (DMSO) from Pierce Chemical Co., Rockford Ill., and agitated by a magnetic stir bar for 24 h. After centrifugation, two mL of the supernatant of each sample were saved and frozen. The pellets from the DMSO extraction were washed twice with deionized water and the supernatant discarded. The insoluble material that remained was resuspended in deionized water, frozen and lyophilized with the yield (dried wall material) recorded.

Separation of hemicellulosic components

One hundred mg of dried wall material (duplicates) were placed into 30 mL Corex test tubes. This material was extracted sequentially with 10 mL of 0.1 M KOH, 20 mL of 1.0 M KOH, and 4.0 M KOH, each containing 3 mg/mL $NaBH_4$ to prevent elimination of the reducing end of the polysaccharides by the alkali (Aspinall *et al.*, 1962). Each extraction was done twice and carried out under nitrogen gas (N_2) for about 1 h at room temperature. The KOH supernatants were filtered by aspiration through Whatman GF/F glass fiber mats (Fisher Scientific, Itasca, Ill., USA), chilled in an ice bath, and the filtrate neutralized drop-wise with glacial acetic acid. About one-tenth of the total volume of each KOH supernatant was saved for analysis of uronic acid by a carbazole method (Dische, 1947) modified by addition of sulfomate (Galambos, 1967). The

remainder was dialyzed against running deionized water for 18-24 h, frozen and lyophilized.

Determination of cellulose content

The cellulose content of the material remaining after KOH extraction was determined gravimetically after hydrolysis in acetic-nitric acid as described by Updegraff (1969).

Determination of neutral sugar content

For analysis of neutral sugar composition, duplicate samples containing about 1 mg of dry wall material were placed in 1-dram glass vials and hydrolyzed in 2 M trifluoroacetic acid (TFA) containing 1 μmol of myo-inositol (internal standard) for 90 min at 120°C. The TFA was evaporated by adding 1 mL of tert-butyl alcohol while under a stream of N_2. Sugars in the residue were reduced and acetylated according to a procedure modified (Carpita and Whittern, 1986) from that of Blakeney *et al.* (1983). Sugars were dissolved in 100 μL of NH_4OH and 0.5 mL of 20 mg/mL $NaBH_4$ in DMSO (w/v) and incubated at 45°C for 90 min with occasional vortex mixing. The solution was neutralized with 100 μL of glacial acetic acid. A 100 μL aliquot of 1-methylimidazole (Sigma) was added, followed by 0.5 mL of acetic anhydride, and the mixture was incubated at room temperature for 30 min.

Water (1.5 mL) was added to destroy unreacted acetic anhydride, and when cool the alditol acetates were partitioned (twice) into 0.5 mL of

dichloromethane. The dichloromethane extractions were collected with a Pasteur pipet and transferred to new vials and dried under a stream of N_2 at 40°C. The entire procedure was completed in 1-dram vials sealed with Teflon-lined caps.

Derivatives were separated by gas-liquid chromatography on a 0.25 mm x 30 m fused silica column of SP-2330 (Supelco, Bellafonte Penn., USA) with temperature programmed from 170 to 240°C at a rate of 5°C/min and then held at 240°C for 6 min. Injection port and flame ionization detector (FID) were at 250°C. Samples of 2 μL were injected with a split ratio of about 1:5. The flow of the Helium (He) carrier was approximately 1.5 mL/min.

Cell wall methylation analysis

For linkage analysis, samples were methylated by a modification (Shea *et al.*, 1989) of the method of Kvernheim (1987). Duplicate samples of cells (approx. 1.0 mg) were placed in 15-mL Corex (Corning Glass from Fisher Scientific, Chicago, Ill.) tubes containing a stir-bar; they were sealed with two perforated layers of Kimwipe paper tightened with small rubber bands and desiccated with the appropriate number of teflon-silicon seals in a vacuum desiccator over P_2O_5 overnight (11 h). The tubes were removed from the desiccator and sealed immediately with their respective seals. They were then evacuated by piercing the stopples with a small gauge syringe needle connected to a vacuum pump via tubing for about 2 min.

Ultrapure N_2 (Matheson Gas, Chicago) was introduced by means of a syringe needle into a bottle containing anhydrous DMSO (Pierce). Under

pressure, 1 mL of anhydrous DMSO was drawn and added to each sample by means of the syringe needle. The tubes were sonicated at 50°C for 3 h in a water bath.

Ultra-high purity N_2 (Matheson) was introduced by means of a syringe needle with a second needle inserted for escape flow. An aliquot of 500 uL of 2.5 M n-butyllithium in hexanes (Aldrich Chemical Co., Milwaukee, Wis., USA) was added to each tube. After the solution had stirred for 3 h under continuous N_2 flow, 500 μL of methyl iodide (Fisher) were added to each tube. The N_2 flow was stopped and about 8 mL of deionized water along with 2.5 mL of chloroform were added to each tube. The tubes were sealed and left stirring overnight at ambient temperature.

The per-*O*-methylated polymers were recovered using procedures described by Carpita and Whittern (1986). This consisted of a continuous partitioning of polysaccharides with deionized water and chloroform with sequential washing with deionized water followed by recovery by centrifugation. The per-*O*-methylated polymers were then hydrolyzed in 2 M TFA for 90 min at 120°C. The mixture was cooled to 30°C, and TFA was evaporated under a stream of N_2. The per-*O*-methylated sugars were reduced with $NaBD_4$ and acetylated according to the method of Blakeney *et al.* (1983). The per-*O*-methylated alditol acetates were separated by a Hewlett Packard 5970 Mass Selective Detector (MSD) with a 0.25 mm x 30 m vitreous silica wall-coated open-tubular capillary column of SP-2330 (Supelco). Temperature was

programmed from 160 to 210°C at a rate of 2°C/min then from 210 to 240°C at 5°C/min. It was held at 240°C for 10 min.

Samples in dichloromethane were introduced by means of a Varian split/splitless capillary injector operated in the split mode (split ratio 50:1). The injector temperature was 225°C. Helium (He) was used as the carrier gas at 180 kPa. The interface oven temperature was 240°C, and the ion source temperature was 300°C. Mass spectra were recorded at 70 eV with an emission current of -0.4 mA and A/V sensitivity of 10^{-7}. The scan time was 0.95 s (0.05 s reset) over the m/z range of 41-350. The derivatives were identified by comparison with spectra of standards prepared from p-nitrophenylglycosides according to Carpita and Shea (1989).

Cell wall uronic acid content

Total uronic acid content was estimated by a carbazol method (Dische, 1947) modified by addition of sulfamate (Galambos, 1967) with a mixture of galacturonic acid and glucuronic acid as standard.

To identify and quantify proportions of galacturonic acid, glucuronic acid and 4-methyl glucuronic acid, derivatives of 6,6-dideuteriohexitols of carboxyl-reduced glycosyluronic acids were prepared by a procedure modified (Carpita, 1989) from that of Taylor and Conrad (1972). Reduction of the uronic acid group was determined only in the 0.1 M KOH fraction because this fraction was enriched in uronosyl residues from the cell wall material.

About 10 mg of wall material were placed in 50 mL beakers containing 10 mL of deionized water. While stirring, the pH of the mixture was kept at 4.7 by adding, drop-wise, 0.1 or 0.001 M HCl solutions. Then 0.5 g of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluelensulphonate (CMC, Sigma) was added to each sample. The mixture was stirred for about 2 h maintaining the pH at 4.7 using the HCl solutions described above. The beakers were then chilled in an ice bath and 3 mL of ice-cold 4 M imidazole-HCl buffer were added. The pH was adjusted to 7.0 using concentrated HCl and stirred for about 15 min. Two hundred mg $NaBD_4$ (3x) were added directly to the solution and the pH was permitted to rise to 8.5 over 1 h. Drops of octyl alcohol were added to halt foaming in the samples. Excess $NaBD_4$ was destroyed with glacial acetic acid. The material was then dialized against running deionized water for 24 h, frozen and lyophilized.

Identification and quantitation of 6,6-dideuterio derivatives

Carbodiimide-activated reduction of the carboxyl groups of glycosyluronic acids with $NaBD_4$ results in an easily identified tag that provides not only diagnostic analysis, but also quantitative determination of the proportion of the uronic acid relative to its corresponding neutral sugar (Carpita and Shea, 1989).

These sugar derivatives were not resolved chromatographically. Hence, mass spectral analysis was the means of identification and quantitation after using the alditol acetate method of Albersheim *et al.* (1967) as cited by York *et al.* (1986).

The ratio of rhamnose (Rha), arabinose (Ara), xylose (Xyl) glucose (Glc), and galactose (Gal) was quantified by comparison of their response in GC-MS spectra to a standard mixture of 50 nmoles of each sugar. The ratio of glucuronic acid (GlcA) to Glc and galacturonic acid (GalA) to Gal was estimated by the ratio of their m-59 ions at m/z 377 to m/z 375 for the HexA and Hex respectively.

The amount of 4-methyl glucuronic acid (4-me GlcA) was estimated by integration of the ion at m/z 191. Since a standard of 4-me GlcA was not available, its amount was quantified by an integral analysis in GC-MS by the scanning approach assuming that it gives a similar response in total ion chromatogram to Glc. The response of the ion at m/z 191 (4-me GlcA) was then compared to the response of the Glc ion at m/z 375 to arrive at a relative response factor for the two compounds.

The samples were then run in the selected ion mode, and the ratio of Glc to GlcA was determined directly by the ratio of m/z 377 to m/z 375 as was the ratio of Gal to GalA. The ratio of 4-me GlcA to Glc was estimated by using the ratio of m/z 191 to 375 and the response factor determined as described above.

Cell wall amino acid content

Amino acids were analyzed according to the procedures of Rhodes *et al.* (1981) to derivatize the amino acids. Ten mg of dry wall material were placed into 3 mL vials and hydrolyzed in 1 mL 6 M HCl at 110°C for 18 h. The samples were centrifuged in microfuge tubes at 4000 rpm for 10 min. Aliquots of 0.75 mL

(except 0.5 mL for the cell line 400 mM NaCl) from the supernatant were transferred to 1 mL microreaction vessels. Fifty-μL of 10 mM pipecolic acid were added to each sample as an internal standard and rotary evaporated to dryness redissolved (twice) in 2 mL deionized water, then rotary evaporated to dryness to remove excess HCl at room temperature.

The samples were applied to 2.5 cm x 1.0 cm columns of Dowex-50-H^+ washed sequentially with 6 mL of deionized water. The amino acids were then eluted with 6 mL of 6 M NH_4OH. The eluate was evaporated to dryness and redissolved in 1 mL of 60% (v/v) methanol. Aliquots of 0.5 ml of the samples were transferred to 1 mL microreaction vessels for derivatization.

In addition, 100 μL of a standard mixture of amino acids [each amino acid at 2.5 mM (Sigma, AA-S-18)], 100 μL of AA-S-18 plus 50 μL of 10 mM pipecolic acid and, 100 μL of AA-S-18 plus 50 μL of 10 mM hydroxyproline were placed in 1 mL microreaction vessels for derivatization and derivation of response factors required for quantitation of amino acid levels. One hundred μL of AA-S-18 (Sigma) were equivalent to 250 nmoles of all amino acids (17) contained in it except for cystine which was equivalent to 125 nmoles because of the occurrence of two cysteine residues on it.

All samples, including standard mixtures, were evaporated to dryness under a stream of dried compressed air at room temperature. The samples were further dried with 100 μL aliquots of methylene chloride with a single repetition. Aliquots of 0.1 mL of a freshly prepared solution of isobutanol-HCl (0.4 mL of acetyl chloride mixed with 2 mL of ice-cold isobutanol in a sealed vial at 4°C with

continuous stirring) were added to each vial. They were sealed with cap and Teflon coated septum, vigorously mixed, and then heated at 110°C for 20 min. After cooling, excess isobutanol-HCl was evaporated under a stream of compressed, dried air, and 50 μL of heptafluorobutyric anhydride were added. The vials were again sealed with fresh septa and heated at 110°C for 10 min. After cooling, the samples were evaporated to dryness under a stream of dry air and were finally dissolved in 100 μL of ethyl acetate: acetic anhydride (1:1, v/v).

Amino acids from wall hydrolysates were converted to the N-heptafluorobutyryl isobutyl ester derivatives. They were separated by injecting 1 μL of each sample into a Varian model 3700 gas chromatograph equipped with a fused silica capillary nonpolar column of SE30 (0.2 mm x 30 m) (Supelco). The temperature program included 4 min at 100°C, followed by an increase to 260°C at a rate of 6°C/min. Injection port and FID temperatures were each 280°C. The carrier was He at a pressure of 10 p.s.i. (corresponding to a flow rate of about 30 mL/min).

Quantitation of the relative proportion of individual polysaccharides

Each fraction of the cell wall contained a mixture of polysaccharides. To deduce polymer structure from linkage analyses a procedure similar to that described by Carpita (1984) was used. To deduce the structure of individual polysaccharides based solely on linkage analysis *a priori* requires some assumptions. Fortunately, there is reliable information on the major hemicellulosic constituents purified from grasses such that some reasonable assumptions can

be made (Darvil *et al.*, 1980; Wilkie, 1979). Since the total wt percent contribution of the three KOH fractions (hemicelluloses) (Table 3) as well as the linkage mol percent (Table 9, 10, 11) are known, the hemicellulosic linkages may be subgrouped into three major fractions based on the known polymers found in grasses and cereals (Burke *et al.*, 1974; Darvill *et al.*, 1980; Carpita *et al.*, 1985 Labavitch and Ray, 1978). Highly substituted glucuronoarabinoxylan (HS-GAX) was considered composed of all the t-arabinosyl, t-galactosyl, 4- and 3,4-xylosyl units of the 0.1 M KOH fraction. Glucuronoarabinoxylans (GAX) were considered composed of the same linkage units, but as the total from that in the 1.0 M and 4.0 M KOH fractions. Xyloglucans were considered comprised of all t-xylosyl, 2-xylosyl, and 4- and 4, 6-glucosyl units. Pectic linkages were separated into two groups based on the known polymers found in grasses (Burke *et al.*, 1974; Carpita, 1985). For galactans, all the galactosyl units were considered for their relative quantification. Arabinans were considered to be composed of all 2-arabinosyl, 3-arabinosyl, and 5- and 3,5-arabinosyl units.

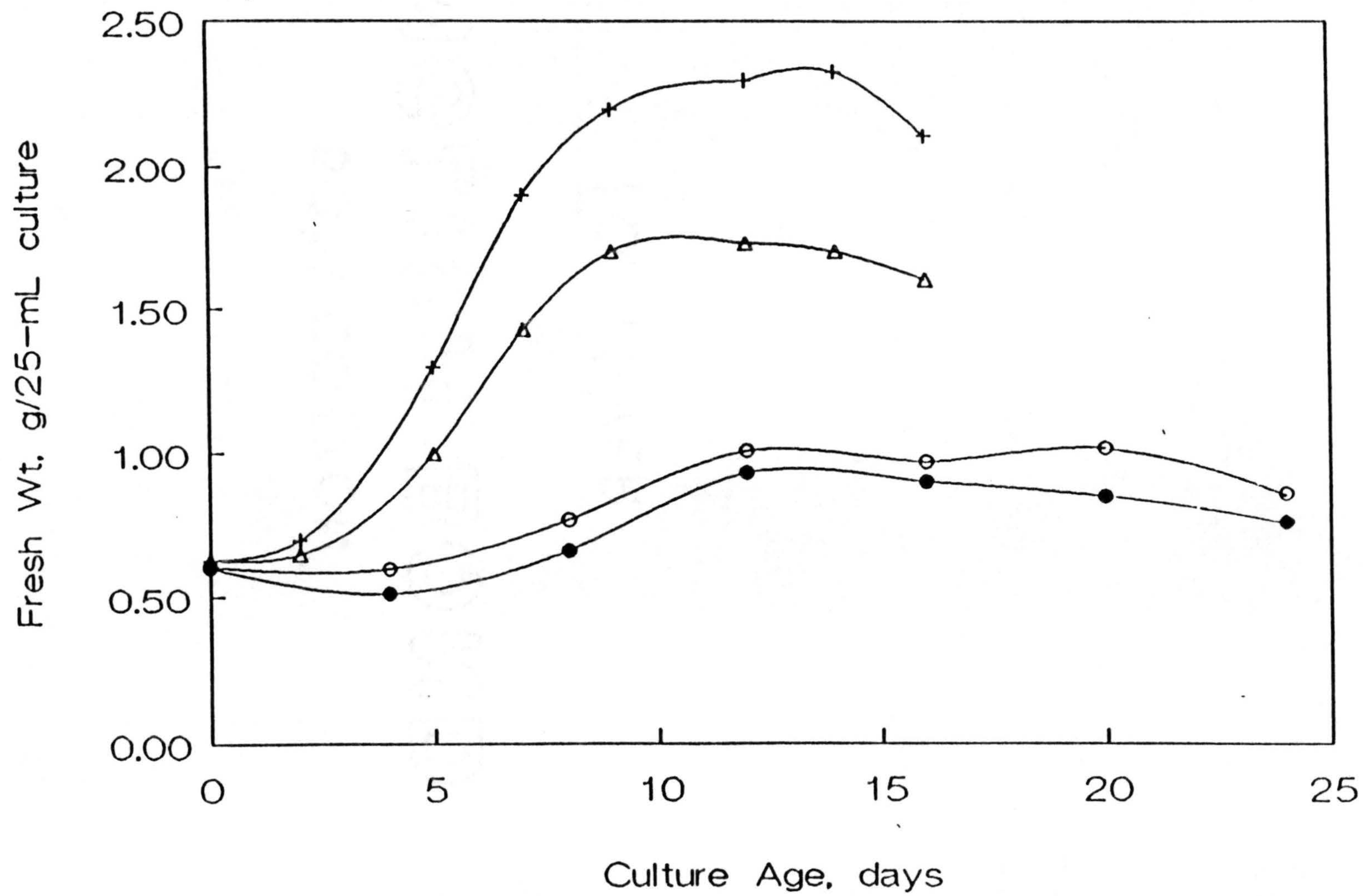
As indicated earlier, to compute these estimates it required that the total weight of the wall material and the proportion of that weight that was hemicellulose (Table 3), as well as the linkage mol percent (Table 9) had to be known. For example, HS-GAX in unadapted cells was 23.7% (t-ara) + 4.2% (4-xyl) + 36.2% (3,4-xyl) + 26 (t-gal) = 66.6% of the total 0.1 M KOH fraction, which is 11.5% of the total KOH soluble fraction [8.4 : (8.4 + 33.2 + 31.7)]. Therefore, 33 HS-GAX = 7.66% of the soluble polymers.

RESULTS

Changes in volume of cultured cells adapted to high levels of NaCl and PEG

Unadapted cells exhibited a typical growth curve upon subculture to fresh medium; for example, a lag phase of 2 d followed by a rapid growth phase until about 9 d when the cells entered stationary phase (Figure 1). The cell lines that were adapted and grown in a medium with 200 mM NaCl or 20% PEG showed similar patterns of growth, but tended to gain fresh weight at reduced rates compared to unadapted cells (Figure 3). The final fresh weight accumulation in the adapted cell lines was reduced in comparison with that in the unadapted cell line, and the decrease in growth was relatively proportional to the level of adaptation (Figure 1). Cells grown either in 400 or 500 mM NaCl had greatly reduced fresh weight accumulation (Figure 1). However, their rates of dry weight gain were slightly lower with respect to the unadapted cells; in fact, the final dry weight gain at stationary phase (after 15 d) was similar for all cell lines (Figure 2). The 400 and 500 mM NaCl cell lines also exhibited a slightly longer lag phase (5 d) as compared to the unadapted cells. This indicates that these cell lines may not be completely adapted to these salt conditions, or the cells were actually in a state of stress with respect to fresh weight accumulation.

Figure 1. Accumulation of fresh weight during the culture cycle of unadapted cells (+) and cells adapted to NaCl at 200 (Δ), 400 (○) and 500 mM (●).



2.50
2.00
1.50
1.00
0.50
0.00
0
5
10
15
20
25
Fresh Wt, g/25-mL culture
Culture Age, days

Figure 2. Accumulation of dry weight during the culture cycle of unadapted cells (+) and cells adapted to NaCl at 200 (Δ), 400 (○) and 500 mM (●).

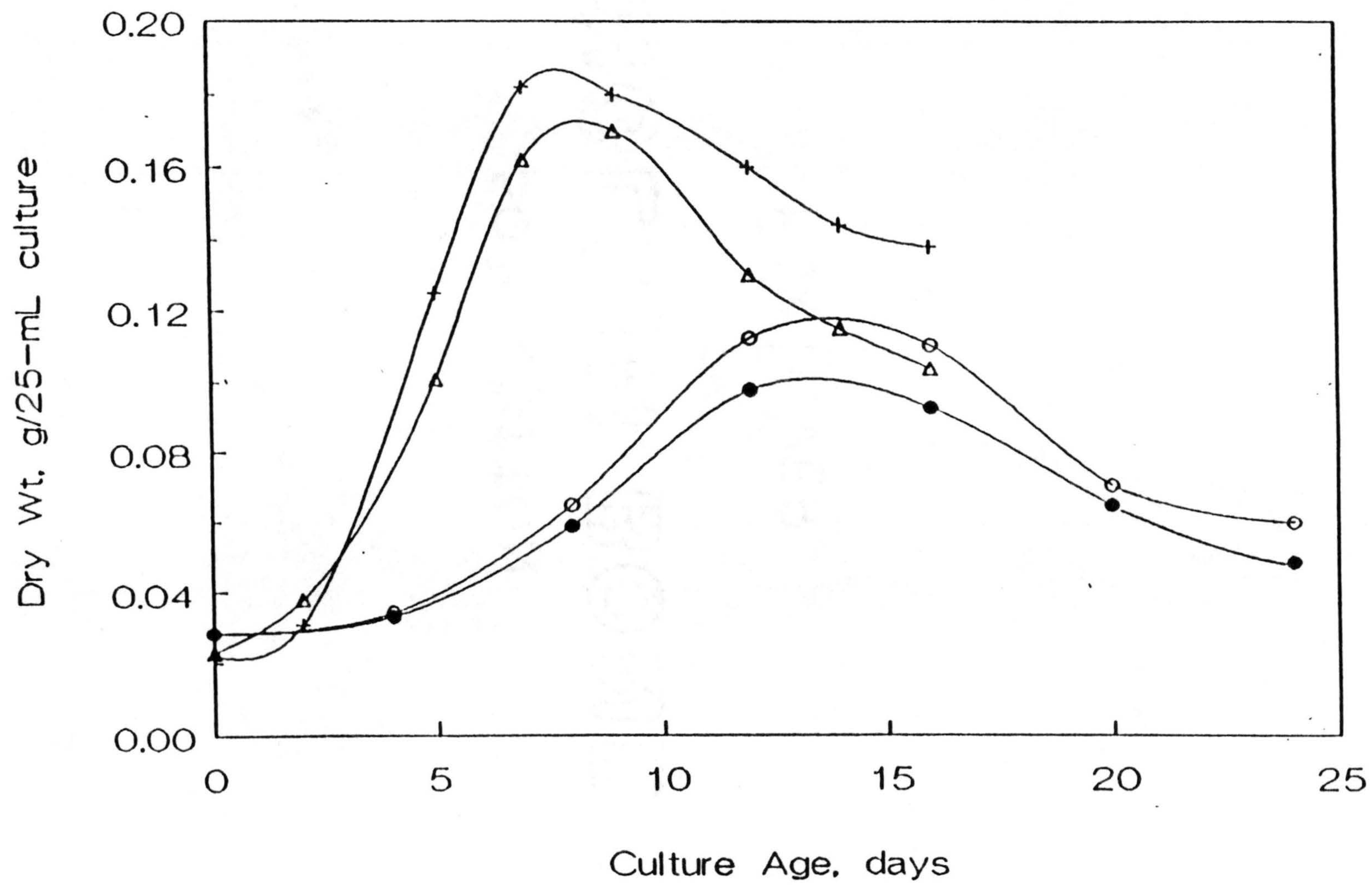
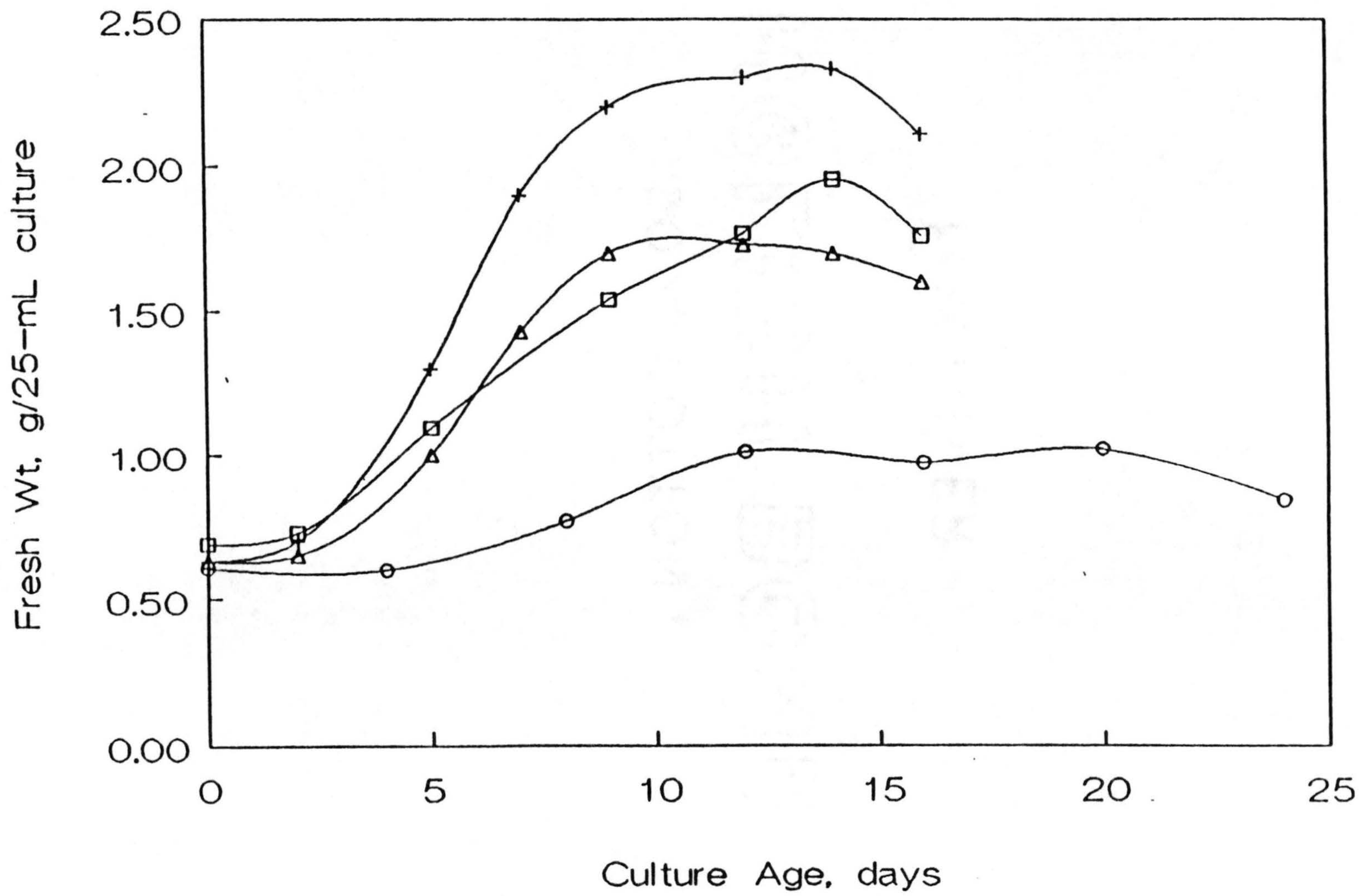

0.20
0.16
0.12
0.08
0.04
0.00
Dry Wt, g/25-mL culture
0
5
10
15
20
25
Culture Age, days

Figure 3. Accumulation of fresh weight during the culture cycle of unadapted cells (+) and cells adapted to NaCl at 200 (Δ) 400 mM (○) and cells adapted to 20% PEG (□).



2.50
2.00
1.50
1.00
0.50
0.00
Fresh Wt, g/25-mL culture
0
5
10
15
20
25
Culture Age, days

Unadapted cells of *D. spicata* are small in comparison with those of tobacco (Iraki *et al*., 1989a), adapted cells have measurably smaller dimensions as well (Table 2) and a discrete rate of cell enlargement (Figure 4). Maximum cell volume of cells in 200 and 500 mM NaCl is about two thirds and one-half respectively of that of unadapted cells (Figure 5).

Indirect evidence that turgor pressure changes in cells adapted to osmotic stress

Since the cells are assumed to be in equilibrium with the medium to which they are adapted, the water potential of the cells was taken to be equal to that of the medium. The water potential of the latter was measured at the end of the cell cycle in all cases as described in the Materials and Methods.

The osmotic potential of unadapted and adapted cells up to 500 mM NaCl was measured by determining incipient plasmolysis (Figure 6) using NaCl and sucrose as plasmolytic agents. Incipient plasmolysis occurred at -17.5 bars for unadapted cells; -31.0 bars for cells in 200 mM NaCl; -43 bars for cells in 400 mM NaCl and -53.0 bars for cells in 500 mM NaCl. Since cells were in a water potential equilibrium with the culture medium, the -3.5 bars water potential of medium without NaCl indicates a turgor pressure of +14 bars for unadapted cells when they are subcultured into fresh medium free of NaCl and +20 bars for cells in 200 mM NaCl, when they are subcultured into fresh medium containing 200 mM NaCl which has a water potential of -11 bars. Similar procedures were

performed to compute turgor pressures of cells grown in 400 mM and 500 mM NaCl. The results are shown in Table 2.

Table 2. Calculation of the tensile strength of unadapted and NaCl - adapted *D. spicata* cells.

Cell line	Turgor pressure[a]	Breaking pressure[b]	Cell Size[c] length	Cell Size[c] width	Tensile strength[d]
	bar	bar	μm		bar
Unadapted	14±3	68±4	58±8	39±5	8,000 -11,900
200 mM NaCl	20±3	44±6	45±3	35±2	5,600 - 7,200
400 mM NaCl	23±2	34±4	40±3	29±2	4,132 - 5,700
500 mM NaCl	29±3	14±2	36±2	28±1	3,010 - 3,870

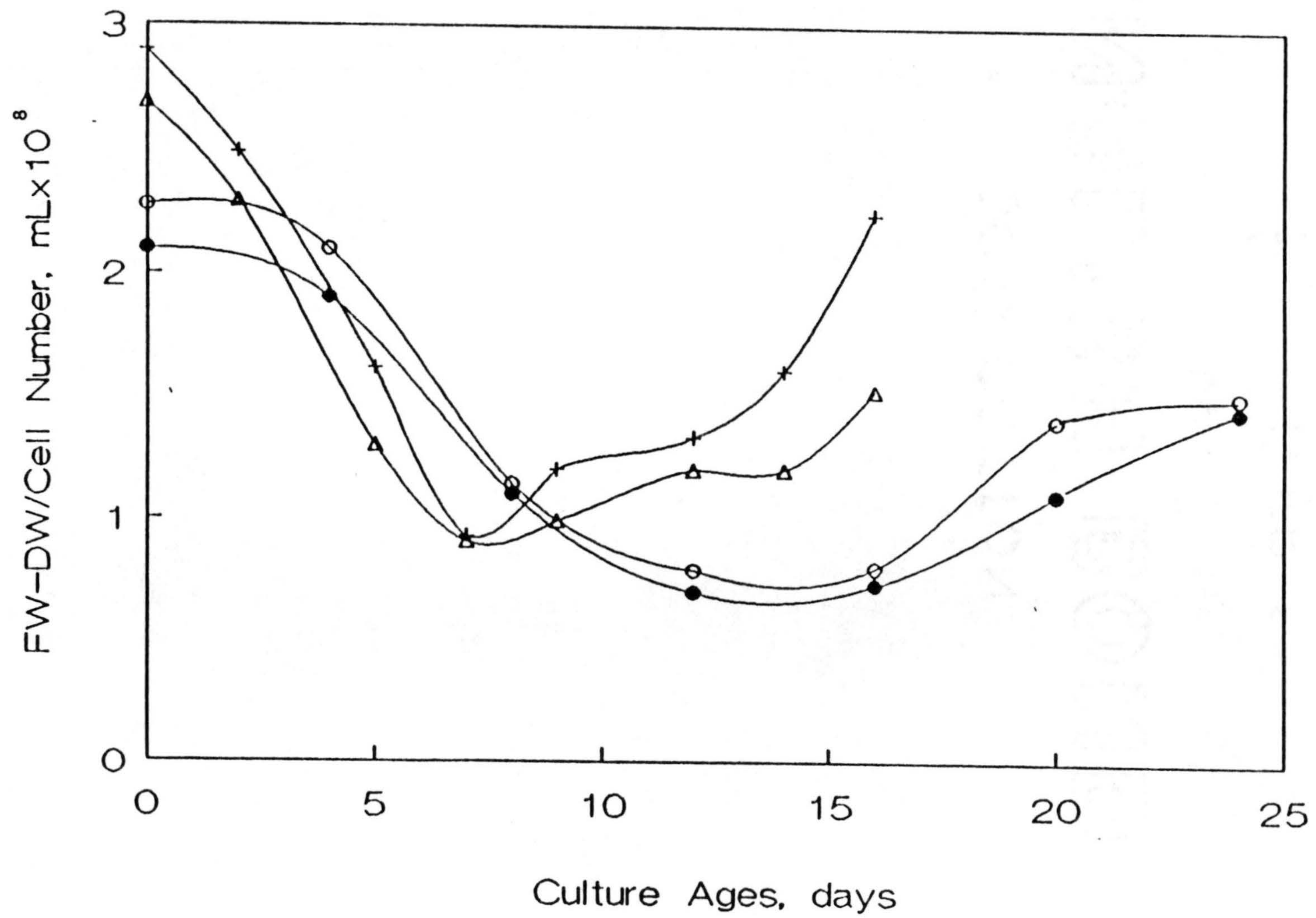
[a]Calculated from incipient plasmolysis of 50% of the cells in a graded series of NaCl and sucrose, and the water potential of the incubation medium.

[b]Pressure in excess of turgor pressure required to burst 50% of the population of cells by nitrogen gas decompression. Values represent means of at least three experiments.

[c]Lengths and widths of ellipsoidal and spherical cells were measured empirically in a population of cells at stationary stage of growth. Values represent the mean of at least 40 samples.

[d]Tensile strength was estimated using the equation (P.r)/2t, which reflects the difference in area of the cell and cell wall upon which the force is applied: P is the sum of the turgor and the breaking pressure, r is the radius estimated from length and width, and t is wall thickness (0.1 μm Halperin and Jensen 1967; Fry, 1988). The range denotes the difference in length and width.

Figure 4. Changes in cell volume during growth of unadapted cells (+) and cells adapted to NaCl at 200 (Δ), 400 (o) and 500 mM (•). Volume was estimated from determination of fresh weight minus dry weight of an aliquot of the cell culture with subsequent calculations of cell number. Enough cells were counted in each aliquot to provide a variance less than ± 5%.


FW-DW/Cell Number, mL x 10^8
3
2
1
0
0
5
10
15
20
25
Culture Ages, days

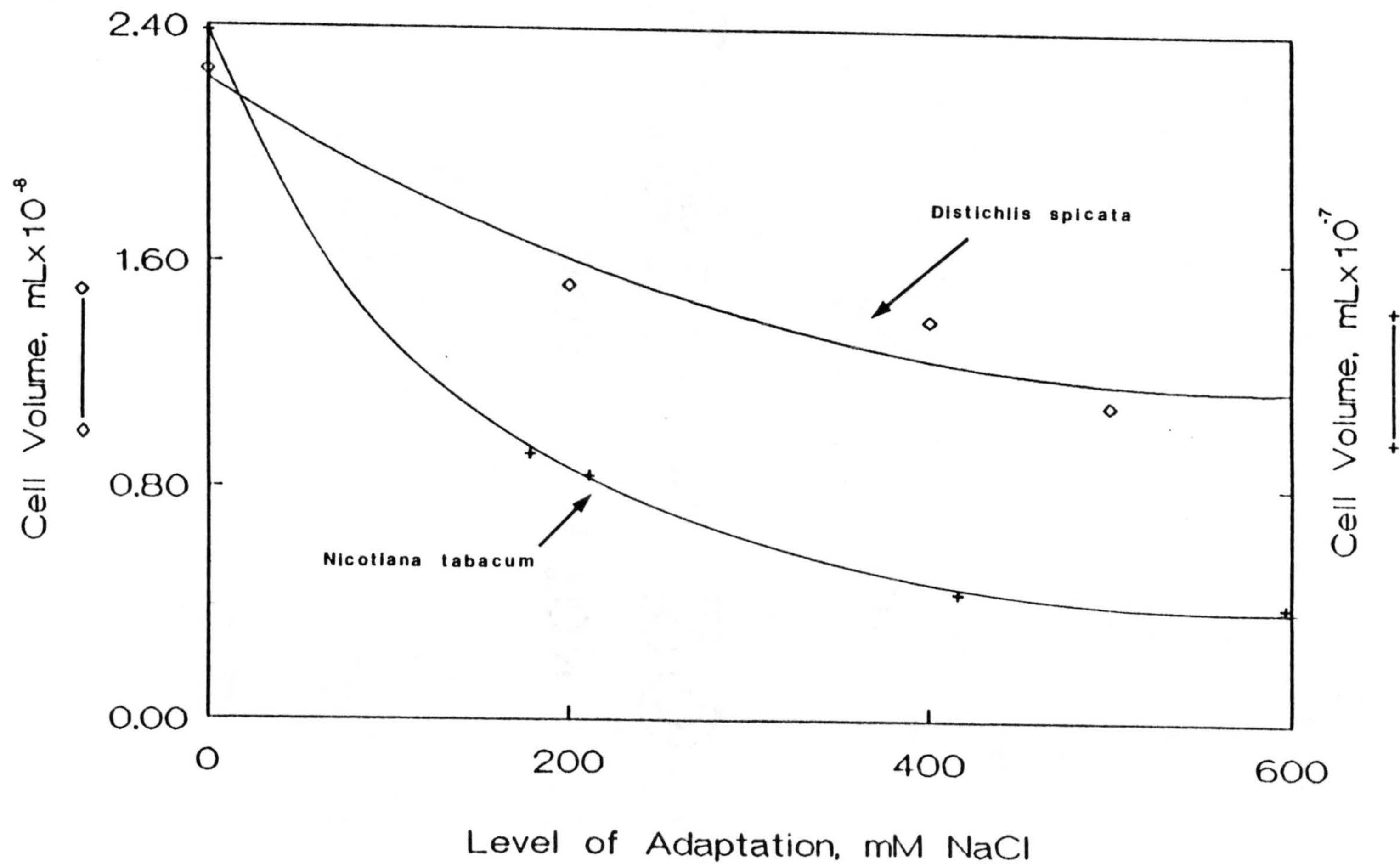


Figure 5. Comparison of the cell volume of NaCl-adapted cells of *D. spicata* and *N. tabacum* (Iraki *et al.*, 1989a). Values are average cell volumes of cell populations at stationary growth phase.

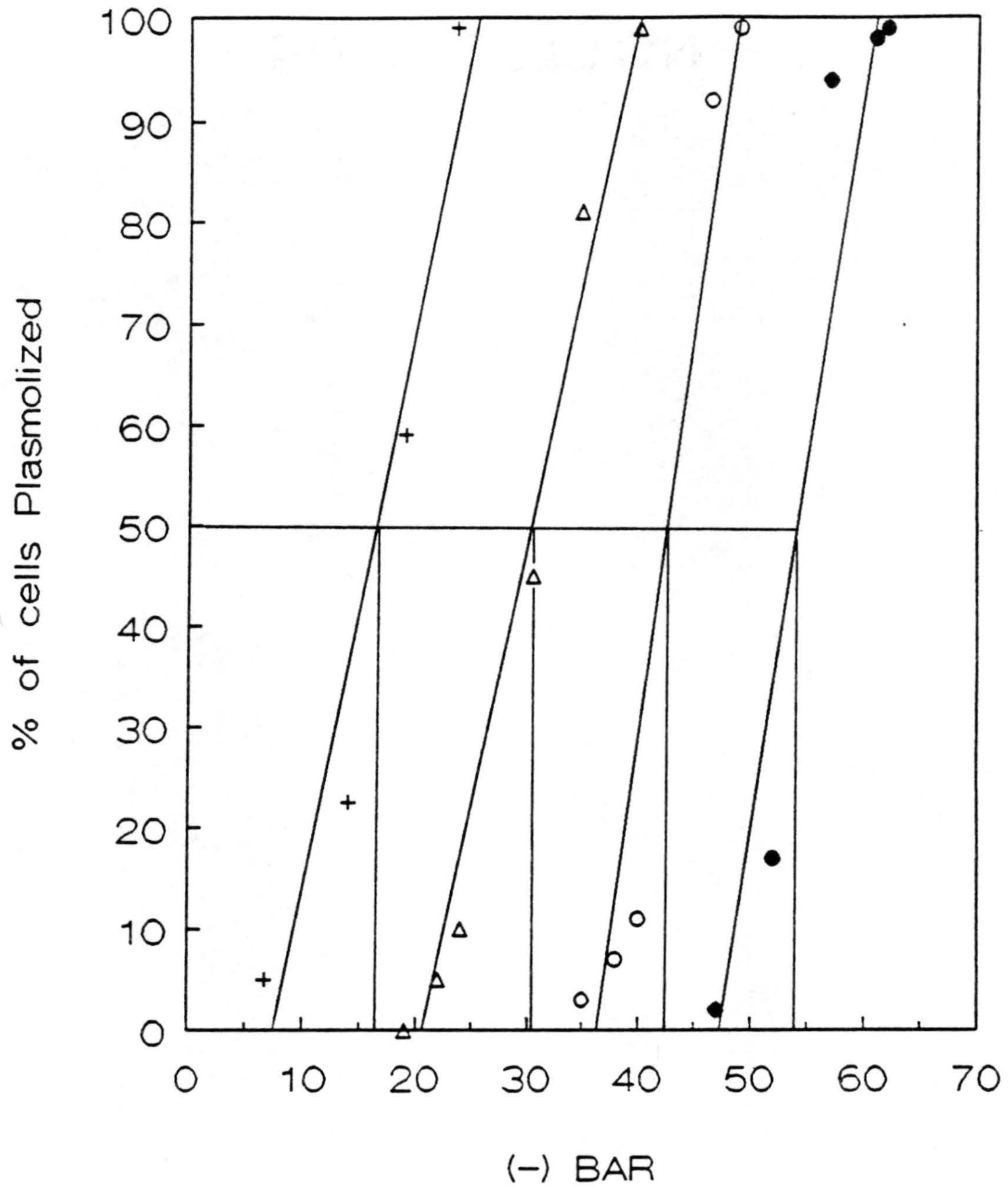


Figure 6. Percentage of cells plasmolyzed in NaCl and sucrose of unadapted (+) and cells adapted to NaCl at 200(Δ), 400 (o) and 500 mM (•). About 200 cells at the stationary growth phase were used for each treatment.

As indicated previously (Materials and Methods), there was wide variation within the cell lines regarding their plasmolytic behavior. Of several hundred unadapted and adapted cells examined, none could be found which was not plasmolyzed by the external lower osmotic potential caused by the plasmolytic agent being used. The difference between the osmotic potential of the cells and the external osmotic potential was consistently in the range of -2 to -3 bars when incipient plasmolysis occurred. This narrow margin is observed in the steep curves shown in Figure 6.

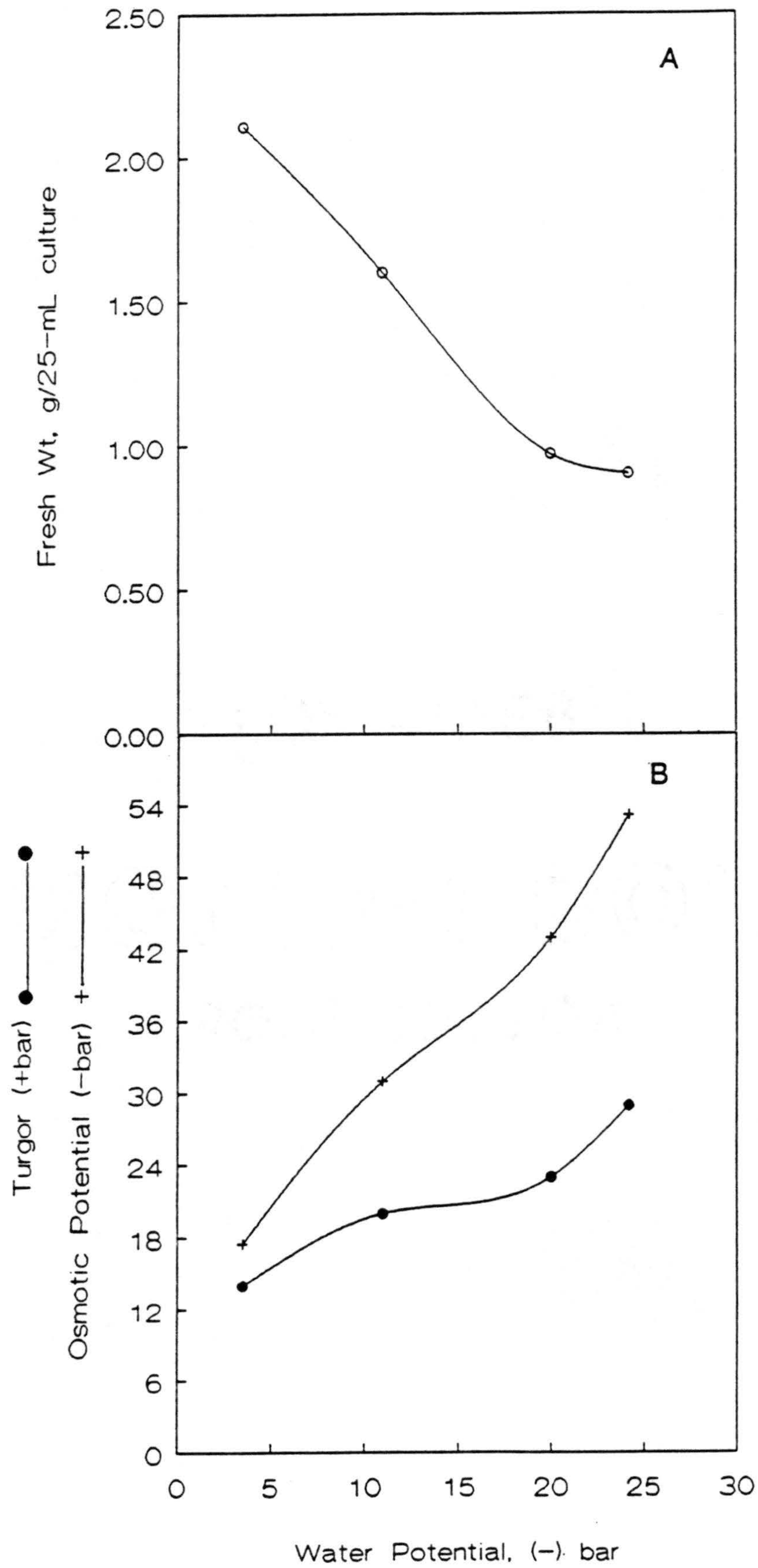
In the cells adapted to media with water potential values of -3.5 bars (free of NaCl), -11 bars (200 mM NaCl), -20 bars (400 mM NaCl) and -25 bars (500 mM NaCl), the average osmotic potential values were -17.5, -31, -43 and -53.2 bars respectively. The average turgor pressure values of the cells increased with the degree of adaptation to salt stress (Table 2), the increase in turgor being especially marked in cells grown in 500 mM NaCl. However, since the total fresh weight accumulation decreased with the level of adaptation (Figure 7), growth was reduced in spite of increases of turgor.

Alteration of cell wall tensile strength associated with salt stress

Tensile strength is the ability to withstand the tangential force per unit wall thickness resulting from the cell's internal pressure. This force may be estimated from breaking pressure, cell diameter, and wall thickness (Carpita, 1985).

Pressures required to break 50% of the population of cells were measured empirically (Table 2). Breaking pressures above turgor pressure were variable

Figure 7. Final fresh weight of cells adapted to the ψ indicated (A), which corresponds to the culture medium free of NaCl and 200, 400 or 500 mM NaCl with average ψ_π and ψ_p (B) as functions of water potential. The average of ψ_π of the culture medium was determined at the stationary growth phase of the different cell lines. The average ψ_p of the cells was determined as described in the Materials and Methods.


A
Fresh Wt, g/25-mL culture
2.50
2.00
1.50
1.00
0.50
0.00
B
Turgor (+bar)
Osmotic Potential (-bar)
54
48
42
36
30
24
18
12
6
0
0
5
10
15
20
25
30
Water Potential, (-) bar

among the unadapted and adapted cells up to 500 mM NaCl (Figure 8). Those for unadapted and cells grown in 200 mM NaCl were about 68 and 44 bars, respectively, while those of cells grown in 400 mM were about 34 bars, and those in 500 mM NaCl were only about 14 bars (Figure 8). Accounting for the contribution of turgor pressure, 82 bars and 64 bars are required to break the walls of unadapted and cells adapted to 200 mM NaCl, respectively. While 57 and 43 bars are necessary to break walls of cells adapted to 400 and 500 mM NaCl respectively (Table 2). Wall thickness was assumed to be 0.1 μm in all cases. Although discrete, cell dimensions were reduced accordingly with the level of adaptation; for example, the average percent length reduction were about 22.4%, 31%, and 37.7% for cells adapted to 200, 400 and 500 mM NaCl, respectively, as compared with the length and width of unadapted cells (Table 2). The estimated tensile strengths were sizable as compared to the breaking pressures, with a range from about 8,000 to 11, 900 bars for unadapted cells and 3,010 to 3,870 bars for cells adapted to 500 mM NaCl (Table 2).

Alteration of the fundamental composition of walls of cells adapted to high levels of NaCl and PEG

The hemicellulosic substances (KOH-soluble material), non-cellulosic material resistant to alkali extraction (acetic-nitric-soluble) and cellulose are compared in Table 3. The KOH-extractable material accounted for as much as 82.9% (500 mM NaCl) of the weight of the wall material at the stationary growth phase as compared to 73.3% of unadapted cells. Interestingly, higher

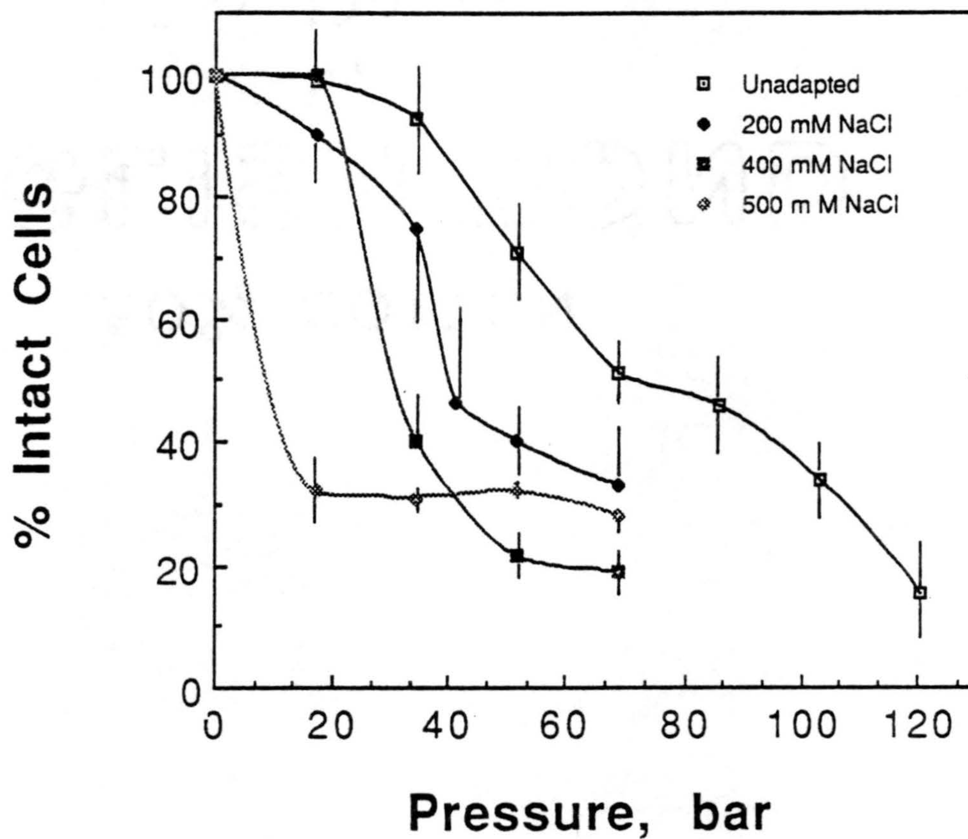


Figure 8. Effect of pressure differential on percentages of intact cells of *D. spicata* adapted to increasing levels of NaCl. Cells were adapted as in Figure 5; pressure differential was generated with nitrogen. Vertical bars are the standard error of measurements from three experiments.

proportions of hemicellulosic polymers were loosely held (0.1 M and 1.0 M KOH soluble) due to salt adaptation; apparently, at the expense of more tenaciously bound polymers (4.0 KOH M and acetic-nitric-soluble). As the salt stresss increased, the amounts of KOH-extractable materials also increased, but amounts of cellulose slightly decreased. In contrast, water deficit (PEG) apparently did not affect the amounts of KOH-extractable material (Table 3). The relative wall mass per cell of NaCl-adapted cells remained much the same, whereas the relative wall mass per cell of PEG-adapted cells was drastically reduced (Table 4). The reason for the loss of wall mass per cell observed in PEG-adapted cells is unknown. Perhaps it was caused by residues of PEG present in the dry cells, thus altering the actual weight of the dry cells when this material was weighed for the cell wall isolation.

The neutral sugars and uronic acids in wall polysaccharides of cells adapted to osmotic stress

The neutral sugar and uronic acid content of the cell walls adaptated to different concentrations of NaCl and PEG-8000 are compared in Tables 5, 6, and 7. These results were obtained from the three KOH fractions ranging from 0.1 M, 1.0 M, to 4.0 M. For the analysis of neutral sugars: fractions from KOH extractions corresponding to those shown in Tables 5, 6 and 7 were neutralized with glacial acetic acid, dialyzed against runing deionized H_2O and separated by GC. Uronic acid was assayed according to a modified carbazole method as described in the Materials and Methods. The identity of the uronosyl residues present in the 0.1 M

KOH soluble fraction of walls of cells adapted to osmotic stress are shown in Table 8. These sugars (Table 8) were analyzed by GC-MS of the prepared alditol acetates of the dideuterio carboxyl reduced cell wall fraction, as described in the Materials and Methods.

In the case of PEG-adaptation the three fractions yielded a remarkable consistency in the neutral sugars and uronic acid proportions. But in the case of NaCl-adaptation some minor differences in constituents could be seen; apparently, only in the 0.1 M KOH fraction. Examination of Table 5 showed that amounts of glucose tended to decrease as the level of salt adaptation increased. Galacturonic acid content is often taken as a reliable indicator of pectic substances in wall chemical analysis. Table 8 showed a decrease of about 50% of GalA in cells adapted to 500 mM NaCl as compared to unadapted cells.

Table 3. Fractional composition of the cell walls from stationary growth phase of *D. spicata* adapted cells to increasing levels of NaCl and PEG-8000. Values represent the average percentage weights of two separate experiments.

Fraction	Unadapted	NaCl (mM)			PEG (%)	
		200	400	500	20	25
		% wt of cell walls				
KOH-soluble						
0.1 M	8.4	8.9	10.9	15.0	12.8	10.9
1.0 M	33.2	35.2	35.6	42.4	33.4	34.3
4.0 M	31.7	33.2	30.4	25.5	26.1	29.0
Sum	73.3	77.3	76.9	82.9	72.3	74.2
Acetic-nitric-soluble	11.1	8.6	9.6	5.9	14.0	9.5
Cellulose	15.4	13.6	13.2	11.0	17.2	13.4

Table 4. Cell wall yields from stationary growth phase cells of *D. spicata* adapted to increasing levels of NaCl and PEG-8000. Values represent the average weights (mg) ± the range of two separate experiments using 2 g of dry cells.

Unadapted	NaCl (mM)			PEG (%)	
	200	400	500	20	25
647±21	533±18	639±18	626±14	390±18	353±21

Table 5. Composition of neutral sugars and uronic acid of KOH-soluble polymers from cell walls of stationary growth phase of *D. spicata* adapted to increasing levels of NaCl and PEG. The extraction was made with 0.1 M KOH. Values represent the mean +/- SD of determinations of at least two independent wall extractions. Values are mol % of the sugar recovered with omission of the uronic acid. Uronic acid values indicate nmoles/mg of cell wall and represent the means of duplicate samples with variance always less than +/- 5%.

Cell type	Arabinose	Xylose	Galactose	Glucose	Uronic Acid
	mol %				
Unadapted	42±3	43±0	5±0	11±2	132
200 mM NaCl	47±3	44±0	6±1	5±2	91
400 mM NaCl	42±2	47±3	5±0	5±1	137
500 mM NaCl	47±3	46±1	6±1	3±0	250
20% PEG	39±0	41±2	4±0	12±3	155
25% PEG	40±0	50±2	4±0	6±3	152

Table 6. Composition of neutral sugars and uronic acid of KOH-soluble polymers from cell walls of stationary growth phase of *D. spicata* adapted to increasing levels of NaCl and PEG. The extraction was made with 1.0 M KOH. Values represent the mean +/- SD of determinations of at least two independent wall extractions. Values are mol % of the sugar recovered with omission of the uronic acid. Uronic acid values indicate nmoles/mg of cell wall and represent the means of duplicate samples with variance always less than +/- 5%.

Cell type	Arabinose	Xylose	Galactose	Glucose	Uronic Acid
	----------	----------	mol %	----------	
Unadapted	38±0	42±0	6±0	14±0	114
200 mM NaCl	36±2	42±0	7±0	15±2	109
400 mM NaCl	41±3	44±0	6±1	11±2	155
500 mM NaCl	41±1	44±3	7±0	10±3	226
20% PEG	37±3	50±0	5±0	18±3	147
25% PEG	39±3	51±0	4±0	6±2	158

Table 7. Composition of neutral sugars and uronic acid of KOH-soluble polymers from cell walls of stationary growth phase of *D. spicata* adapted to increasing levels of NaCl and PEG. The extraction was made with 4.0 M KOH. Values represent the mean +/- SD of determinations of at least two independent wall extractions. Values are mol % of the sugar recovered with omission of the uronic acid. Uronic acid values indicate nmoles/mg of cell wall and represent the means of duplicate samples with variance always less than +/- 5%.

Cell type	Arabinose	Xylose	Galactose	Glucose	Uronic Acid
	----------	----------	mol %	----------	
Unadapted	38±0	43±0	7±0	12±0	244
200 mM NaCl	36±3	45±0	7±1	12±2	221
400 mM NaCl	38±2	41±0	7±0	13±1	235
500 mM NaCl	41±3	38±0	8±1	13±2	232
20% PEG	38±1	46±0	7±0	8±0	216
25% PEG	37±0	47±0	5±0	10±1	240

Table 8. Mole % of uronosyl residues of total glycosyl residues present in walls of cells of *D. spicata* adapted to increasing levels of NaCl. Cell wall fractions extracted with 0.1 M KOH was used to identify and estimate amounts of uronic acid as described in the Materials and Methods.

Cell type	Galacturonic acid	Glucuronic acid	4-*O*-methyl glucuronic acid
		mol %	
Unadapted	4.1	2.1	6.9
200 mM NaCl	1.8	1.4	4.9
400 mM NaCl	2.0	2.0	5.5
500 mM NaCl	2.4	1.8	5.4

Indirect evidence of wall polysaccharide changes with osmotic stress

The actual cell wall polysaccharide proportions can be estimated by glycosyl linkage analysis (Carpita, 1984; Shea *et al.,* 1989). Briefly, based on the reliable work done with grasses and cereals (Burke *et al.,* 1974; Wilkie, 1979; Carpita, 1983; 1984; Carpita and Kanabus, 1988), it is safe to assume that arabinoxylans are the major components in these species. Hence, the highly substituted glucuronoarabinoxylans (HS-GAX) were considered to be composed of all the t-arabinosyl, t-galactosyl, 4- and 3,4-xylosyl units of the 0.1 M KOH fraction (Table 9). Glucuronoarabinoxylans (GAX) were considered to be composed of the same linkage units, but total from 1.0 M and 4.0 M KOH fractions (Tables 10 and 11). Xyloglucans were considered to be comprised of all t-xylosyl, 2-xylosyl and 4- and 4,6-glucosyl units in all three fractions. For galactans, all the galactosyl units were

Table 9. Distribution of neutral sugar linkages in KOH-soluble wall polymers of fractions of stationary growth phase of *D. spicata* cells adapted to increasing levels of NaCl and PEG. The extraction was made with 0.1 M KOH. Aliquots of about 1 mg from each sample in duplicate were permethylated, and partially methylated alditol-acetate derivatives were prepared. Derivatives were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry. Values are in mole % and are the mean of duplicates with variance less than +/- 7%.

Sugar	Glycosidic linkage	Unadapted	NaCl (mM)			PEG (%)	
			200	400	500	20	25
Arabinose	t-ara f[a]	23.6	12.2	31.2	11.7	14.0	4.2
	2-ara f[b]	2.5	2.2	1.8	2.0	2.1	1.4
	3-ara f	7.0	6.6	5.5	7.1	8.8	8.0
	5-ara f	4.4	5.2	2.8	4.9	4.3	2.5
	3,5-ara f	1.7	3.2	1.5	3.3	1.7	2.5
Xylose	t-xyl p	2.9	3.7	5.5	3.9	4.6	1.8
	2-xyl p	1.0	1.9	1.5	1.6	2.0	1.7
	4-xyl p	4.2	7.6	3.1	4.7	5.1	7.3
	3,4-xyl p	36.2	42.9	26.6	41.0	38.8	50.9
Galactose	t-gal p	2.6	3.2	2.0	4.8	3.9	2.4
	3,6-gal p	2.0	1.0	0.0	0.5	1.0	0.0
	4-gal p + 3-glc p	1.1	1.5	14.0	1.6	1.4	1.1
Glucose	t-glc p	1.7	0.9	1.4	2.1	1.4	3.0
	4-glc p	6.2	5.3	0.9	5.5	7.1	8.2
	4,6-glc p	1.3	1.1	0.9	2.3	1.8	3.0
Rhamnose	2,4-rha	0.6	1.0	0.7	2.3	0.7	1.2

[a]t-, nonreducing terminal sugar.

[b]2-ara f, (1→2)-Linked arabinofuranosyl unit deduced from 1,2,4,-tri-*O*-acetyl-(1-deuterio)-3,5-di-*O*-Methyl pentitol identified by GC-MS (Carpita and Shea, 1989).

Table 10. Distribution of neutral sugar linkages in KOH-soluble wall polymers of fractions of stationary growth phase of *D. spicata* cells adapted to increasing levels of NaCl and PEG. The extraction was made with 1.0 M KOH. Aliquots of about 1 mg from each sample in duplicate were permethylated, and partially methylated alditol-acetate derivatives were prepared. Derivatives were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry. Values are in mole % and are the mean of duplicates with variance less than +/- 7%.

Sugar	Glycosidic linkage	Unadapted	NaCl (mM)			PEG (%)	
			200	400	500	20	25
Arabinose	t-ara f[a]	24.4	20.1	13.2	12.3	8.4	10.3
	2-ara f[b]	1.9	1.8	2.0	1.9	2.5	2.0
	3-ara f	5.8	6.5	8.2	8.0	8.8	9.3
	5-ara f	3.8	5.2	6.0	6.6	5.0	4.3
	3,5-ara f	3.7	4.5	4.8	6.7	3.3	1.9
Xylose	t-xyl p	6.0	5.6	4.7	5.5	4.6	5.3
	2-xyl p	2.1	1.7	3.2	2.5	3.7	2.6
	4-xyl p	4.4	7.1	3.8	4.1	7.2	9.2
	3,4-xyl p	27.8	26.4	30.7	23.7	34.2	36.6
Galactose	t-gal p	3.4	4.6	5.7	5.4	4.7	4.2
	3,6-gal p	0.6	0.9	0.6	0.6	0.6	0.5
	4-gal p + 3-glc p	4.4	7.0	2.2	2.5	2.5	1.9
Glucose	t-glc p	1.5	1.7	1.9	4.6	2.3	0.8
	4-glc p	6.4	4.3	7.8	9.1	7.4	7.3
	4,6-glc p	1.7	1.7	2.8	3.6	3.9	2.6
Rhamnose	2,4-rha p	0.9	1.1	1.8	2.5	1.6	0.6

[a]t-, nonreducing terminal sugar.

[b]2-ara f, (1→2)-Linked arabinofuranosyl unit deduced from 1,2,4,-tri-*O*-acetyl-(1-deuterio)-3,5-di-*O*-Methyl pentitol identified by GC-MS (Carpita and Shea, 1989).

Table 11. Distribution of neutral sugar linkages in KOH-soluble wall polymers of fractions of stationary growth phase of *D. spicata* cells adapted to increasing levels of NaCl and PEG. The extraction was made with 4.0 M KOH. Aliquots of about 1 mg from each sample in duplicate were permethylated, and partially methylated alditol-acetate derivatives were prepared. Derivatives were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry. Values are in mole % and are the mean of duplicates with variance less than +/- 7%.

Sugar	Glycosidic linkage	Unadapted	NaCl (mM)			PEG (%)	
			200	400	500	20	25
Arabinose	t-ara f[a]	7.6	11.5	16.1	7.2	11.5	9.3
	2-ara f[b]	1.6	1.2	1.3	1.5	2.7	2.3
	3-ara f	6.0	7.6	8.3	7.7	7.7	19.6
	5-ara f	4.1	5.7	8.0	7.2	4.8	7.3
	3,5-ara f	4.7	6.6	6.8	7.2	4.0	3.3
Xylose	t-xyl p	3.2	7.2	6.8	5.00	6.7	5.8
	2-xyl p	1.7	3.2	1.8	5.3	3.3	3.7
	4-xyl p	2.4	4.8	6.2	5.3	4.2	5.0
	3,4-xyl p	30.3	25.3	20.2	17.9	26.5	21.6
Galactose	t-gal p	11.0	5.9	6.1	7.1	7.6	10.3
	3,6-gal p	0.4	0.1	0.1	0.6	0.3	0.2
	4-gal p + 3-glcp	4.5	3.9	2.3	3.1	2.7	1.2
Glucose	t-glc p	0.7	1.4	1.8	1.5	0.8	0.0
	4-glc p	12.4	9.3	8.5	11.6	9.0	5.8
	4,6-glc p	7.8	3.7	3.6	9.2	6.5	3.5
Rhamnose	2,4-rha p	1.1	2.2	1.6	2.9	1.3	1.1

[a]t-, nonreducing terminal sugar.

[b]2-ara f, (1→2)-Linked arabinofuranosyl unit deduced from 1,2,4,-tri-*O*-acetyl-(1-deuterio)-3,5-di-*O*-Methyl pentitol identified by GC-MS (Carpita and Shea, 1989).

considered; and for arabinans all were considered to be composed of 2-arabinosyl, 3-arabinosyl, and 5- and 3,5-arabinosyl units.

The mole per cent of each of the major glycosyl units, as deduced by GC-MS of the partially methylated alditol acetates, is presented for the 0.1 M, 1.0 M, and 4.0 M KOH fractions obtained from walls of cells of *D. spicata* adapted to NaCl and PEG in Tables 9, 10, and 11. Methylation analyses of these fractionated hemicelluloses (KOH-soluble wall material) demonstrated that they comprise polysaccharides similar to those found in hemicelluloses of maize coleoptiles and proso millet cell suspension cultures (Table 12) as reported by Carpita (1983: 1984) and Carpita *et al.* (1985).

D. spicata cell walls contain an enrichment of t-arabinosyl and 3,4-linked xylosyl residues. This suggests that the major polysaccharide component of these primary cell walls is an arabinoxylan comprised of a 1,4-linked backbone with single arabinosyl residues attached to C-3 of some xylosyl residues of the xylan backbone. Also, the presence of 2- and 5-linked arabinosyl and terminal galactosyl residues may indicate the occurrence of some trisaccharide side chains that would indicate a similarity to those described by Buchala *et al.* (1972) in oat stem arabinoxylan. The amount of 3,4- linked xylose appeared very high in many of the salt and PEG fractions, correspondingly, the amount of t-ara f decreased. This may be inaccurate due to the methylation analysis as Tables 5, 6 and 7 showed that the ratio of arabinose to xylose remained remarkably constant regardless of the osmotic adaptation. It is expected that the t-ara f residues were improperly quantified perhaps caused by the GC-MS injection system.

The influence of the saline stress and water deficit upon the relative distribution of polysaccharides in walls of *D. spicata*, as extracted stepwise with increasing concentrations of 0.1 M to 4.0 M KOH, is illustrated in Table 12. The summation of each polysaccharide proportion is presented in Table 13. Apparently, there is a substantial mole percent decrease of GAX whereas HS-GAX increases in walls of cells adapted to 500 mM NaCl as compared to unadapted cells. Likewise, xyloglucan and arabinan tended to increase in mole percent caused by the saline stress while galactan slightly decreased (Table 13). Thus, it is likely that constituents of these primary cell walls changed as adaptation to water stress deficit as saline stress occurs. Other components did not show a change in amounts, for example, GAX and HS-GAX in walls of cells adapted to 20% and 25% PEG, as compared to unadapted cells (Table 13).

In general, these data are in harmony with previous observations (Table 3) in that proportions of hemicellulosic polysaccharides are more loosely held (0.1 M and 1.0 M KOH) after salt adaptation. Therefore, a noncovalent as well as covalent bond modification is very possible between wall polymers caused by saline stress because the amounts of polymers extracted with base differed after adaptation.

Table 12. Distribution of cell wall polymers deduced from linkage analyses in fractions of walls of *D. spicata* cells adapted to increasing levels of NaCl and PEG. The KOH-soluble polymer extraction was made at stationary growth phase. Rationale for assignments of each polymer are given in the Materials and Methods. Values are mole % of total cell wall polymers.

Cell-wall fraction	Unadapted	NaCl (mM)			PEG (%)	
		200	400	500	20	25
	--------	--------	--------	mol %	--------	--------
0.1 M KOH-soluble :						
Xyloglucan[a]	1.31	1.38	1.25	2.40	2.75	2.16
Arabinan	1.80	1.97	1.64	3.13	2.99	2.11
Galactan	0.30	0.28	1.98	0.38	0.48	0.16
HS-GAX[b]	7.66	7.60	8.93	11.30	10.93	9.52
1.0 M KOH-soluble :						
Xyloglucan	7.34	6.05	8.56	10.60	9.05	8.22
Arabinan	6.88	8.14	9.72	11.85	9.05	8.02
Galactan	2.26	3.60	1.30	1.58	1.43	1.11
Glucurono-arabinoxylan (GAX)	24.40	26.50	24.70	23.50	25.20	27.85
4.0 M KOH-soluble :						
Xyloglucan	10.84	10.06	8.06	9.55	9.20	7.33
Arabinan	7.08	9.07	9.64	7.24	6.93	12.56
Galactan	2.12	1.72	0.95	1.13	1.08	0.54
GAX	22.20	20.42	19.20	11.51	18.00	18.08

[a]Proportions of individual polymers were deduced from linkage analysis based on assumptions described in text.

[b]Highly substituted glucuronoarabinoxylan (HS-GAX).

Table 13. Mole % of KOH-soluble polymers present in walls of *D. spicata* cells adapted to increasing levels of NaCl and PEG. The KOH-soluble polymers were extracted at stationary growth phase. Rationale for assignments of each polymer are given in the Materials and Methods.

Linkage group.	Unadapted	NaCl (mM)			PEG (%)	
		200	400	500	20	25
Xyloglucan[a]	19.50	17.49	17.87	22.55	21.00	17.71
Arabinan	15.76	19.18	21.00	22.22	18.97	22.69
Galactan	4.68	5.6	4.23	3.09	2.99	1.81
GAX[b]	46.66	46.92	43.90	34.70	43.20	45.93
HS-GAX[c]	7.66	7.60	8.93	11.30	10.93	9.52

[a]Proportions of individual polymers were deduced from linkage analysis based on assumptions described in text.

[b]Glucuronoarabinoxylan.

[c]Highly substituted glucuronoarabinoxylan.

Protein content and amino acid composition of walls of cells adapted to osmotic stress

The insoluble protein content of the cell wall remained remarkably constant as the cells adapted to increasing concentrations of NaCl (Figure 9) and slightly decreased with PEG adaptation (Figure 10). The amino acid composition of wall proteins did not change much as cells adapt to salt and PEG, except for the hydroxyproline and tyrosine content. As a percent of the total amino acids, hydroxyproline decreased about 3-fold in walls of cells adapted to 500 mM NaCl, whereas it remained relatively constant in walls of cells adapted to 20% and 25%

PEG (Table 14). Tyrosine increased about 2-fold with higher levels of adaptation to salt and PEG, as compared to unadapted cells (Table 14). The amount of hydroxyproline appeared to be correlated with average cell volume (Figure 11).

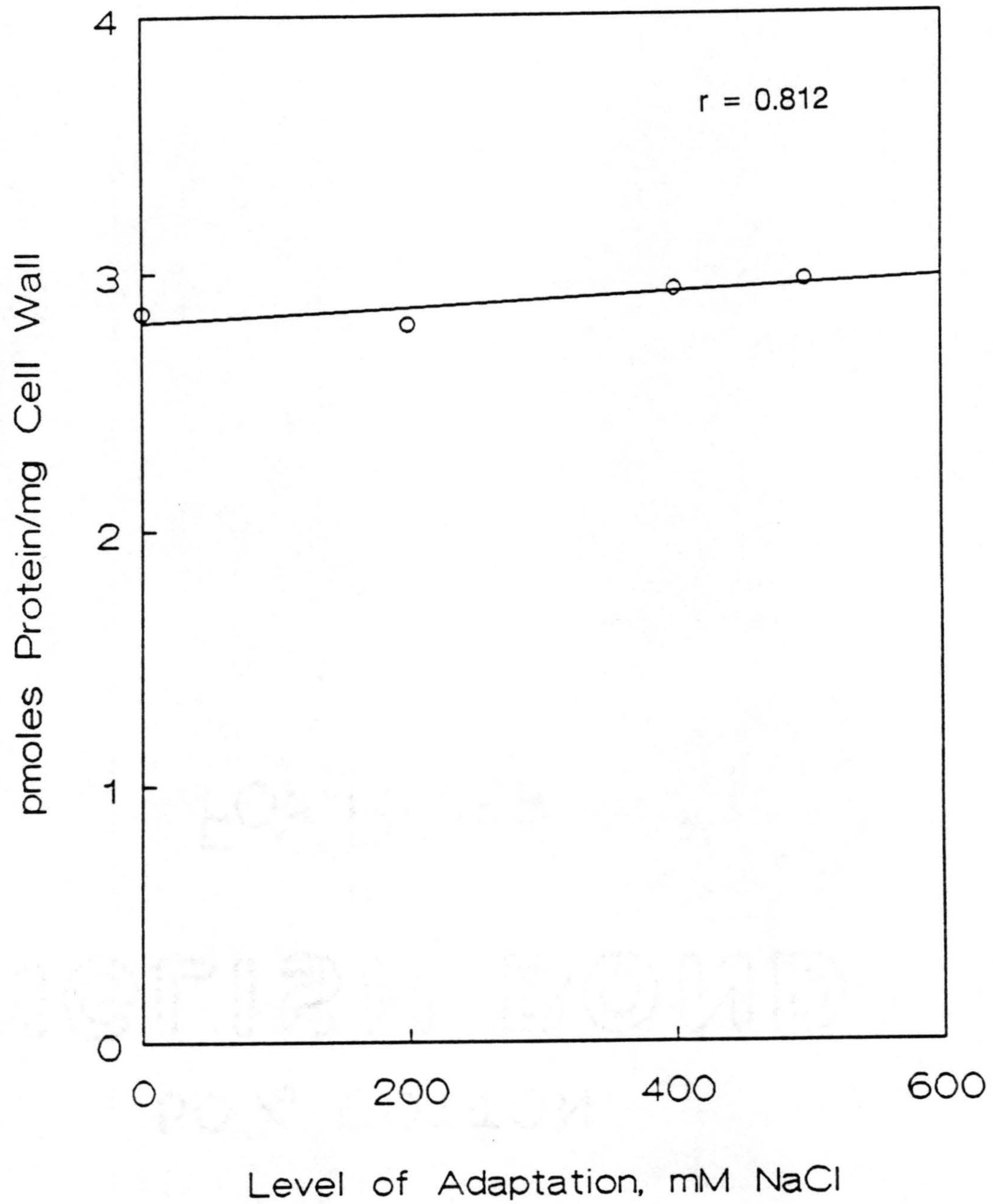


Figure 9. Amount of insoluble protein in cell walls of the stationary growth phase of cells adapted to increasing levels of NaCl. Cells were adapted as in Figure 5; insoluble protein was measured from amounts of amino acids released from 6 M HCl hydrolysis of purified cell walls.

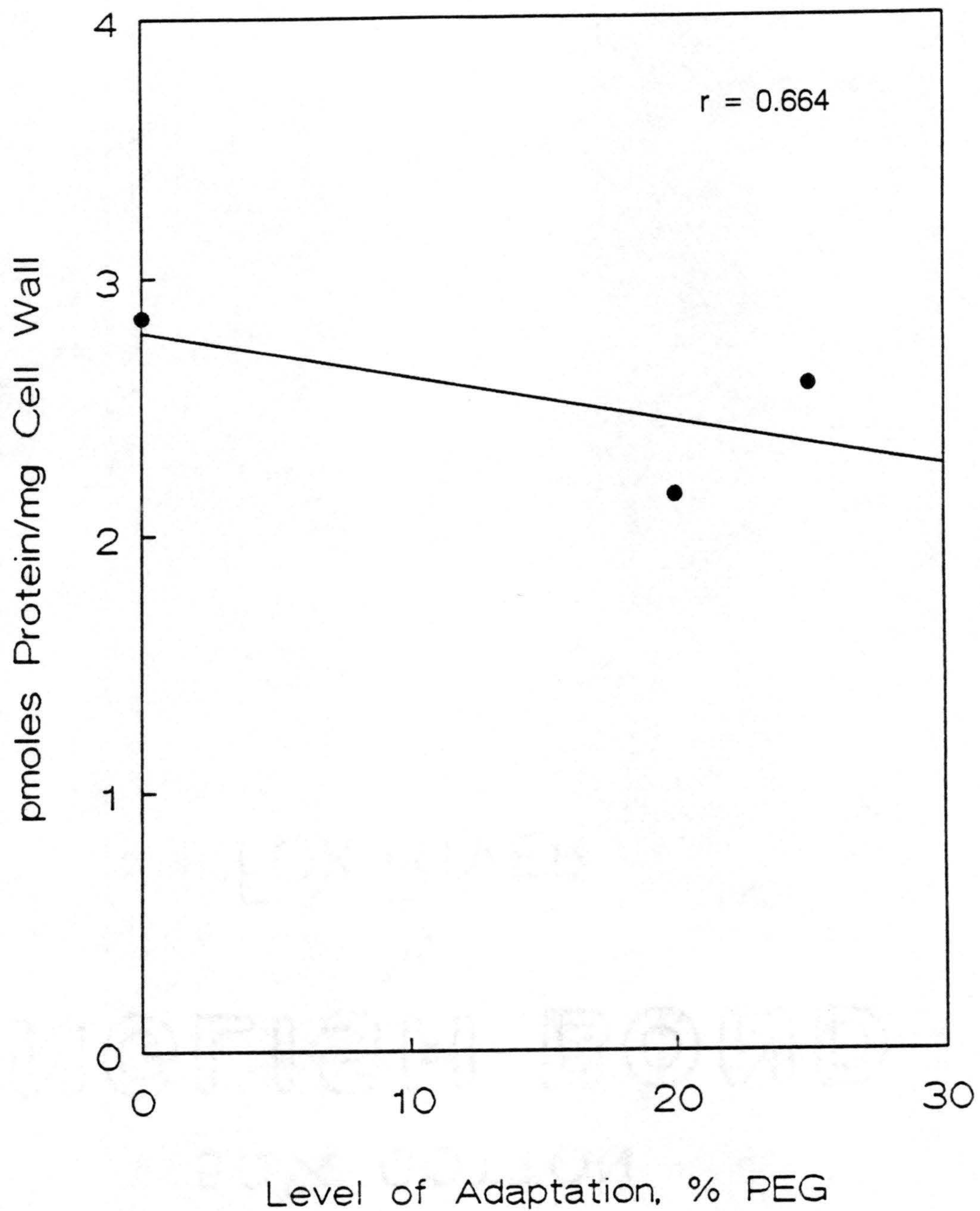


Figure 10. Amount of insoluble protein in cell walls of the stationary growth phase of cells adapted to 20% and 25% PEG-8000. Cells were adapted as described in the Materials and Methods; insoluble protein was measured from amino acids released from 6 M HCl hydrolysis of purified cell walls.

Table 14. Amino acid composition (mole % of total amino acids) of insoluble proteins from cell walls of stationary growth phase of *D. spicata* cells adapted to increasing concentrations of NaCl and PEG-8000.

Amino acid	Unadapted	NaCl (mM)			PEG (%)	
		200	400	500	20	25
	mol%					
Alanine	9.8	10.2	9.9	10.6	10.3	9.4
Glycine	9.2	9.9	11.2	11.7	9.9	9.3
Valine	6.5	10.4	6.5	6.0	5.6	5.9
Threonine	5.5	5.1	5.3	5.3	5.3	5.3
Serine	7.0	7.0	7.6	7.5	7.5	7.2
Leucine	9.7	9.4	9.2	9.3	9.3	9.3
Isoleucine	4.5	4.3	4.0	4.1	3.8	4.1
Proline	5.7	5.2	5.5	5.3	5.8	5.7
Hydroxyproline	**2.0**	**0.9**	**1.0**	**0.8**	**1.8**	**2.6**
Methionine	0.8	0.6	0.8	0.8	1.5	1.2
Asp +Asn	10.7	10.0	10.7	10.5	10.9	10.4
Phenylalanine	3.8	3.6	3.8	3.7	3.5	3.8
Glu + Gln	10.5	10.1	10.3	10.5	10.2	10.2
Lysine	7.5	6.8	6.7	6.4	6.5	7.0
Tyrosine	**1.2**	**1.7**	**2.0**	**2.6**	**2.2**	**2.4**
Arginine	2.1	2.3	1.7	2.3	2.8	2.9
Histidine	2.1	2.0	2.3	2.1	2.2	2.3
Cystine	0.0	0.0	0.0	0.5	0.4	0.4

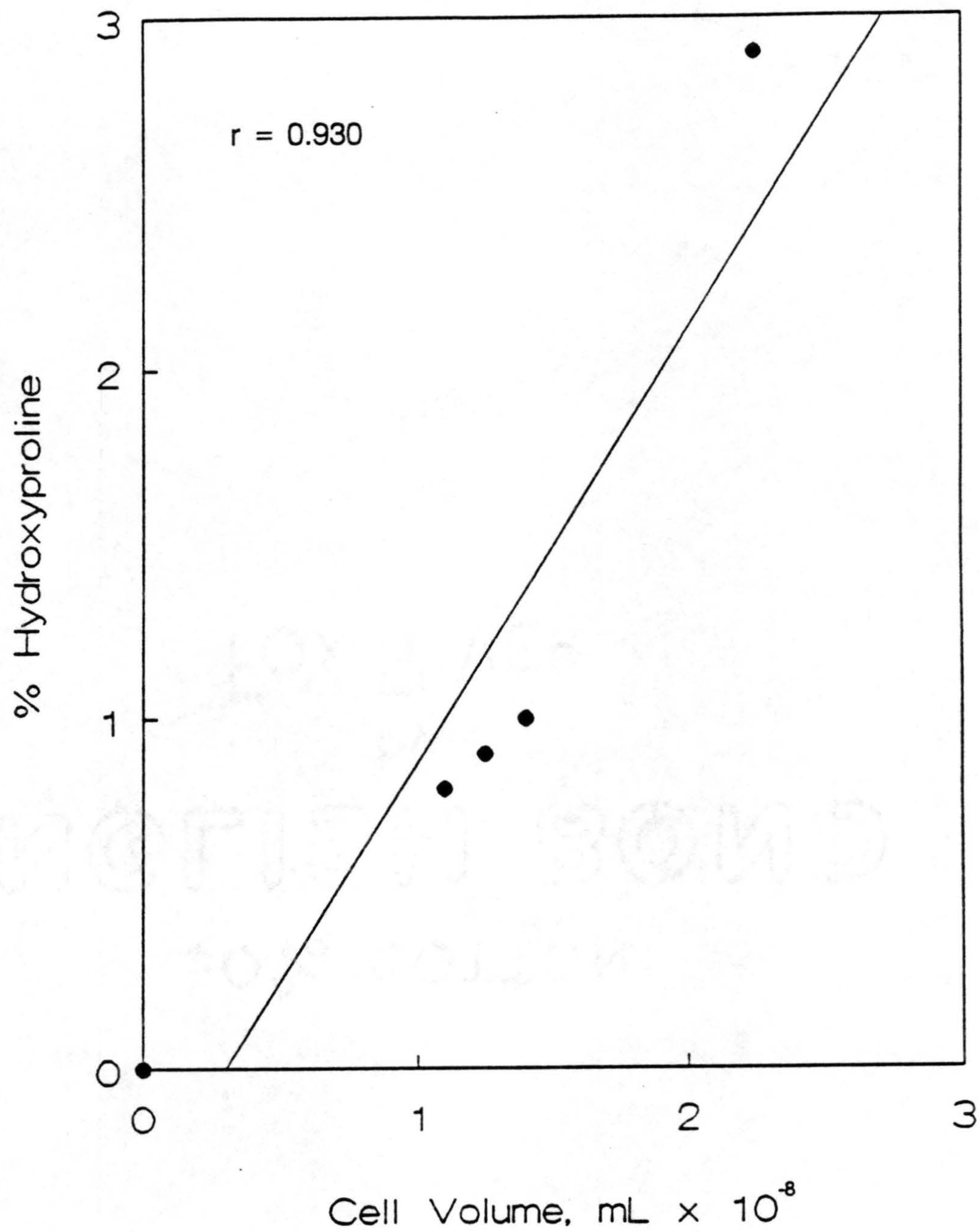


Figure 11. Hydroxyproline content as mole % of insoluble cell wall protein as a function of average cell volume. Cell volume of cells adapted to various levels of NaCl as in Figure 5 and hydroxyproline was measured as in Table 14.

DISCUSSION

Analysis of Growth and cell volume differences

Before commencing this discussion, certain reservations and restrictions must be noted. First, the cellular system used in these experiments is from the monocot halophytic grass, *D. spicata*. Hence, the results generated from this system may not serve as a model for comparison with the results of the dicot (tobacco) system of Iraki *et al*. (1989a; 1989b; 1989c). Second, the length of adaptation to NaCl stress is substantially different between both systems. Tobacco cells were adapted and maintained for approximately 12 years while *D. spicata* adapted cells were used at the end of six months of adaptation. It is believed, however, these differences between the cellular systems do not preclude the use of the salt tolerant cells of *D. spicata* (Warren and Gould, 1982; Daines and Gould, 1985) to either confirm or formulate possible new working hypotheses relative to the morphological wall changes that occur as a result of NaCl stress adaptation, or salt tolerance. In fact, the salt-adapted tobacco cells have shown halophytic characteristics such as Na^+ and Cl^- compartmentalization in the vacuole and proline accumulation in the cytoplasm (Binzel *et al*., 1985; Bressan *et al*., 1985; Binzel *et al*., 1988).

The relationship between turgor and cell expansion rate, and final cell volume are apparently altered by long-term exposure to osmotic stress, although

mechanistically the basis for this alteration remains unknown. Recently, hypotheses have been proposed explaining this reduced growth based on changes in cell wall structure and chemistry of adapted cells of glycophytes (Iraki *et al.* 1989a; 1989b; 1989c; Singh *et al.*, 1989). Just how these hypotheses would be applicable to a halophyte cell system remain unknown but are of great interest.

The results here indicated that cell cultures of *D. spicata,* when adapted (six months) to increasing levels of NaCl, showed decreased cell size despite an increase in cell turgor. Thus, they are less extensible than unadapted cells (Table 2). However, the decrease in cell size is not as drastic (Figure 5) as that of the salt-adapted tobacco cells (Iraki *et al.*, 1989a). The unadapted cells of *D. spicata* were about one order of magnitude smaller than unadapted tobacco cells (Figure 5). From observations by light microscopy, about half of the *D. spicata* cell population was isodiametric and about 50 μm in diameter; these cells grew in clusters generally about 200 μm in diameter containing about 30 cells each. Some clusters of up to 2 mm in diameter formed as the culture approached stationary phase. The ratio of fresh weight to dry weight, which is a rough measure of the extent of cell expansion, was about 7.9 during early logarithmic growth and increased to only 9.5 by stationary phase (data not shown). All these parameters changed when cells adapted to NaCl. In the highest concentration (500 mM) of NaCl, most of the cell population was isodiametric, about 28 μm in diameter (Table 2). The tendency to form clusters and the number of cells contained per cluster was also greater (about 50 cells per cluster). Cell

elongation that predominates during the stationary growth phase in the tobacco cell cultures was not observed in *D. spicata* cultures (Figure 4). These observations are in agreement with those reported from cultures of proso millet (Carpita *et al.*, 1985). Hence, it is likely that in cultured cells of grasses, expansion (a parameter of growth) may be less prominent than it is in cultured cells of dicots. It is interesting to note that the average turgor pressure of unadapted cells of *D. spicata* was about +14 bars whereas the turgor pressure reported for tobacco was only +4 bars. This property may contribute to the salt tolerance of the cultured cells of *D. spicata*, because the gain of fresh and dry weight was just slightly affected when cells were transferred from culture medium free of salt into fresh culture medium containing 200 mM NaCl (Figures 1, 2). At the end of six months growth in 200 mM NaCl the turgor pressure had increased to about 6 bars and about 9 bars and 15 bars in 400 mM and 500 mM NaCl respectively (Table 2). These changes in turgor pressure and osmotic potential clearly demonstrated osmotic adjustment in these cells.

Alteration of the tensile strength as linked to NaCl stress

Only results obtained from unadapted and NaCl-adapted cells will be discussed in this section because the technical problems encountered with cells in the viscous PEG medium hampered the nitrogen gas decompression experiments. As in salt adapted tobacco cells, there was a definite loss of tensile strength in cells of *D. spicata* adapted to different levels of NaCl (Table 2). But, unlike adapted tobacco cells, there was no effect on the relative mass of the cell

wall per cell (Table 4). Tensile strength of primary cell walls, as defined earlier in the literature review, is a parameter distinct from that of extensibility in that tensile strength units would be in N . m^2 or Pa (0.1 MPa is equivalent to 1 bar) which reflects the external force exerted in a single dimension required to break a material; for example, steel rods or plant fibers. The tensile strength determinations reported here are based on the internal pressure required to burst the cells and was estimated from breaking pressure, cell diameter, and wall thickness. Extensibility, on the other hand, is the inverse of viscosity whose units are bar^{-1} s^{-1}, as in longitudinal extensibility. Measurements of wall extensibility *in vitro* have only been reported in pieces of individual giant internodal cells of the algae *Nitella* (Metraux and Taiz, 1978). No higher plant cells are large enough to allow these measurements of extensibility. The alternative approach has been to use whole organs (e.g. stems) or tissues (e.g. epidermal strips) in which the contribution of turgor has been eliminated by plasmolysis by means of freezing/thawing or treatment with boiling methanol. Such specimens can be held in a tensile tester (e.g. Instron) and stretched as their behavior is observed (Cleland, 1981; 1984; 1987).

Since the force applied by the large cell volume is resisted by an extremely thin wall, tension developed within the wall is several orders of magnitude higher than turgor pressure (Table 2). Tensile strength of walls of adapted cells as based on calculations of breaking pressure (Figure 8) and cell geometry is considerably lower (Table 2) in each case. However, the pressure required to break the walls is still much higher than normal turgor pressure. Thus, 'wall

expansion' during growth appeared to be controlled by a discrete rearrangement of the wall matrix and may not be viewed as a critical pressure required to pry the microfibrils apart mechanically. Iraki *et al.* (1989a) proposed a diversion of carbon away from cellulose synthesis and failure of the structural protein extensin to polymerize as the principal factors contributing to a loss of tensile strength in salt-adapted tobacco cells. Results with NaCl-adapted *D. spicata* cells suggest that this hypothesis does not hold true for this halophytic grass since 1) there is relative loss of wall mass per cell from adapted cells (Table 4) and, 2) the wall structure is different. Hydroxyproline decreased about 3-fold, as percent of the total amino acids, in cell walls adapted to 500 mM NaCl. However, the type of protein that contains the hydroxyproline is not known. This is consistent with the salt adapted tobacco cells. Also, a 2-fold increase of tyrosine in walls of cells adapted to higher levels of NaCl and PEG can be observed (Table 14). Interestingly, higher proportions of hemicellulosic polymers loosely held (soluble in 0.1 M and 1.0 M KOH) were substantially affected by salt adaptation. Apparently, this was at the expense of more tenaciously bound polymers (soluble in 4.0 M KOH and acetic-nitric acid) while no effect was observed with PEG (Table 3). Although there is no gross changes in chemical composition of neutral sugars (Tables 5, 6, 7, 9, 10, and 11) either in noncellulosic polysaccharides (Table 13) or in insoluble protein (Figure 9) there were, however, marked alterations in the organization of glucuronoarabinoxylan polymers, perhaps in cross-linking by phenol substances. Therefore, the fundamental question is

which rearrangements within the wall matrix are responsible for the reduction of the wall breaking strength caused by salt stress.

It has been demonstrated that phenolic cross-bridges constitute a major constraint to the fractionation of polymers in the different KOH concentrations (Carpita, 1984; Fry, 1986; Nishitani and Nevins, 1990); hence, strongly suggesting that phenolic residues are linked to arabinoxylans. The major phenolic acid is ferulic acid, and dimerization of ferulic acid could result in polymer cross-linkages (Carpita, 1984). Diferulate residues are known to be present in maize (Carpita and Whittern, 1986) and other cereal plants (Shibuya, 1984; Nishitani and Nevins, 1990).

These results suggest a dynamic adjustment in both glucuronoarabinoxylan and phenolic substances in the wall. The correlation of these events with the altered growth physiology of NaCl-adapted cells, for instance a slight decrease in cell size despite an increase in turgor pressure, implies an involvement in cell expansion of these cultured cells. This interpolymeric alteration is important enough to modify the physical properties of walls of cultured cells of *D. spicata*. This is clearly demonstrated by the reduced tensile strength of NaCl-adapted cells. These findings demonstrate, for the first time, that cellulose microfibrils are not the only determinant that confers tensile strength to the primary cell wall, but rather subtle changes in the matrix polysaccharides are likely responsible for this event, at least in cultured cells of grasses. This conclusion is in general agreement with that reported by Carpita (1984) based on alkali extraction procedures and that of Nishitani and Nevins (1990) based on

enzymic analysis of feruloylated arabinoxylan derived from maize cell walls. Moreover, despite such marked differences in the determinants proposed for tensile strength, these values (Table 2) are comparable to those of the salt-adapted tobacco cells. It should be noted that the chemical bases that would explain differences between the properties of tensile strength and extensibility cannot be readily deduced from this study. NaCl-adapted cells have a reduced ability to expand and have higher turgor pressures even though tensile strength is substantially lower (Table 2). The intriguing 2-fold increase of tyrosine in walls of cells adapted to higher levels of NaCl gives rise to speculation about potential factors contributing to a loss of extensibility. It is plausible that a decrease in extensibility is a result of both a change in the organization of glucurono-arabinoxylan-phenolic substances and perhaps to a partial lignification of the wall, thereby locking together large portions of the innermost layers of the wall. A determination of the composition of material secreted into the culture medium may possible provide important insights to events occurring in the wall matrix. Thus, the discrete biochemical properties of the wall that give rise to tensile strength and the dynamic properties of wall metabolism that give rise to extensibility during cell expansion could be more readily resolved in these cultured cells.

Salt and water deficit (PEG) apparently differ in their effect on the organization of wall constituents of *D. spicata*. This was observed with the glucuronoarabinoxylan-substances that were affected by salt but unaffected by 20% and 25% PEG (-16 and -22 bars, respectively) and, in the mol percent of

hydroxyproline of total amino acids that remained relatively constant in walls of cells adapted to the PEG concentrations, but increased with salt. Additional research to determine the effect of water deficit and salinity on wall morphology of these cultured cells is clearly warranted.

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APPENDIX

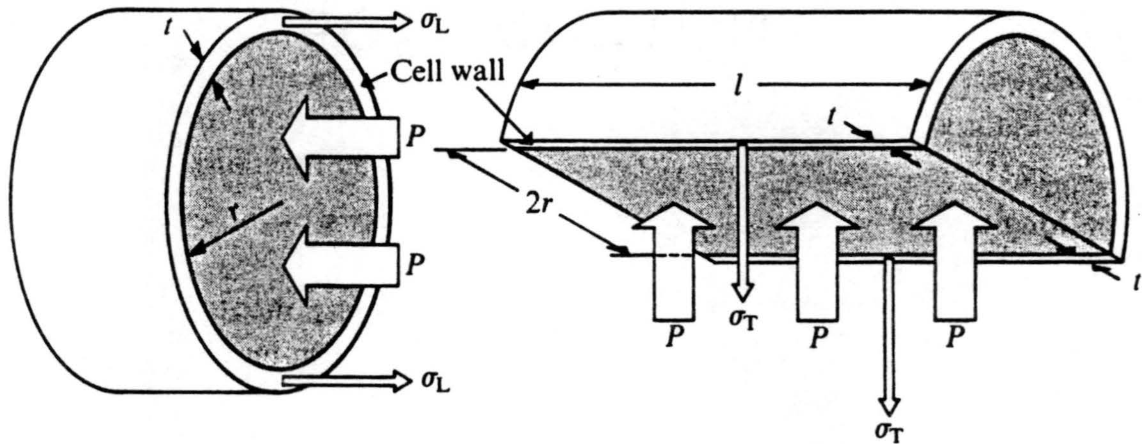

Figure 12. Schematic sections of a hypothetical cylindrical cell illustrating various dimensions and the stresses (σ_L and σ_T) existing in the cell wall. One way to calculate the stresses is to imagine that the cellular contents are removed, leaving only the cell wall, which has a uniform hydrostatic pressure *P* acting perpendicular to its inside surface. The projection of this *P* over the appropriate area gives the force acting in a certain direction, while the reaction to this force is an equal force in the opposite direction in the cell wall. by dividing the force in the cell wall by the area over which it occurs, we can determine the cell wall stress (After Nobel, 1983).

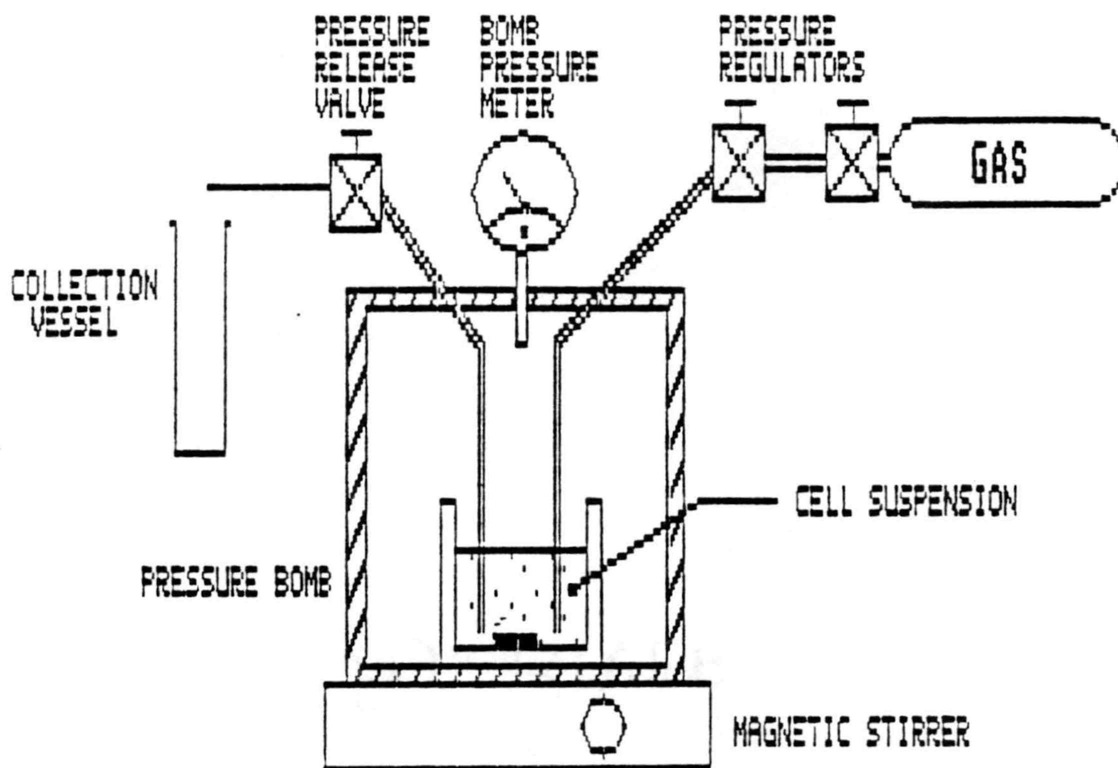

Figure 13. Exploded view of the components of the Parr decompression bomb, as used to determine breaking pressures of primary cell walls.