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ORGANIC AND INORGANIC CONSTITUENTS OF SALT TOLERANT TARO *(COLOCASIA ESCULENTA* VAR *ANTIQUORUM)* TISSUES CULTURED IN SALINE MEDIA

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(*Received* 1 *October* 1986; *accepted in revised form* 6 *February* 1989)

NYMAN L. P., ARDITTI J. and BRADLEY T. J. *Organic and inorganic constituents of salt tolerant taro* (Colocasia esculenta *var* antiquorum) *tissues cultured in saline media.* ENVIRONMENTAL AND EXPERIMENTAL BOTANY **29,** 423–432, 1989.—Salinity tolerant taro tissues were selected and cultured *in vitro* on saline media (50–350 mOsm). Salt tolerance in these tissues was associated with increased levels of calcium oxalate, chlorophyll, protein and some secondary alkaloids. The concentration of other secondary alkaloids as well as the levels of quaternary alkaloids decreased. Calcium, potassium, magnesium and sodium content of the tissues decreased with increased salinity. These findings are discussed in relation to salinity tolerance in taro.

INTRODUCTION

HALOPHYTES and salt tolerant cultivars of several crop plants have been studied extensively in recent years.[7,17,20] These studies suggest that adaptation to salinity may involve a number of mechanisms. One of these appears to be an accumulation or change in the constitution of components that affect the ionic and osmotic balance of cells.[16,28] These components include organic acids, nitrogenous compounds, carbohydrates and mineral ions.[4,5,22,25]

Determining which mechanisms are involved in salt tolerance in previous work is difficult because information has been obtained from diverse experimental systems. These include: (1) several naturally-occurring halophytes, (2) a number of plants selected for salt tolerance from standard lines, (3) cultured tissues, seedlings and field-grown plants, and (4) inter-specific hybrids between halophytes and glycophytes. In addition, plants or cultures were often maintained under various environmental conditions, and with different sources of salinity, as for example, NaCl, natural seawater, artificial seawater or several salt mixtures.[4–7,15,19,22,25,32,33,35]

Improved knowledge of the basis for salt tolerance in callus and in plantlets produced *in vitro* may aid in the development of more direct selection methods. Such knowledge can be obtained from studies involving tissues and/or plants of a single glycophyte selected for tolerance to a graded series of salinity levels. Salt tolerant taro tissues selected in our laboratory[23] are such a system. Using this system we have attempted to limit variables for the purpose of gaining a better understanding of adaptation to salinity by glycophytes.

MATERIALS AND METHODS

Tissue culture

Tissues, including protocorm-like bodies and undifferentiated cell masses,[24] were obtained by

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culturing shoot tips of *Colocasia esculenta* UCI Runner on solid Linsmaier-Skoog medium (Table 1) containing 1 mg/l adenine-*N*-benzyl-tetrahydro-2H-pyran-2yl (SD 8339, Shell Research, Modesto, CA) or 1 mg/l dimethylaminopurine (DMAP) and 0.1 mg/l naphthaleneacetic acid (NAA).[1] Cultures were maintained at $26 \pm 1°C$ under 18 hr photoperiods and light intensity of $2.5 mW/cm^2$.

Salinity

To select for salinity tolerance, tissue sections *ca* 0.1 cm^3 were transferred to successively higher concentrations (5% or 50 mOsm increments) of saline water (SW) approximately every 2.5 months.[29] Sections were taken from the most vigorously growing cultures. At least 20 new cultures were made every time. SW stock solution was prepared by dissolving 13.96 g NaCl, 0.39 g KCl, 2.25 g $MgSO_4 7H_2O$ and 3.80 g $MgCl_2$ in 100 ml of double distilled water.[2] This solution was used at dilutions of 50–350 mOsm (equivalent to approximately 5–35% natural seawater).

Organic analyses

Organic compounds were assayed using methods we have used before.[23] This facilitated direct comparisons between the levels of organic constituents in mature plants and seedlings (Table 2) and cultured tissues. Protein content was determined by multiplying nitrogen levels by 6.25. Nitrogen determinations were obtained with a Coleman Model 29 analyzer.[31] All tissues from two replicate samples were analyzed for each SW concentration. The samples (i.e. culture vessels) were selected at random from among all cultures on a certain SW level.

Calcium oxalate (CaO) was assayed by permanganate titration.[11,31]

Alkaloid concentrations were measured with Wagner's and Mayer's reagents.[8,16] This technique was modified only to the extent that a Klett colorimeter was used to measure turbidity of thoroughly agitated samples.[31]

Chlorophyll content was determined by methods adapted for use with orchid protocorms and employed previously for taro.[31]

Inorganic analyses

Tissues were dried at $50 \pm 2°C$ until there were no further weight losses (usually 3 days), extracted with 10 N HCl and adjusted to an equal volume. Ionic concentrations in tissues were obtained by transferring samples to two dram polyvials (Van Waters and Rogers) containing 1 ml of 0.1 N HCl and the following additives: none for sodium; 2 mg of cesium chloride for potassium; and 10 mg lanthanum chloride for calcium and magnesium. Concentrations were determined using a Varian AA-275 Series Atomic Absorption Spectrophotometer.[26] Three replicates, each consisting of all the tissue in a culture vessel, were used for every SW concentration. Culture vessels for assays were selected at random.

RESULTS

Calcium oxalate

Levels of CaO in all cultured tissues were much lower (regardless of the SW concentrations used) than those in leaves and corms of greenhouse grown plants (Table 2). CaO content in the tissue generally increased with SW concentration (Fig. 1A). Tissues grown on 0 and 50 mOsm had the lowest concentration of CaO and did not differ from each other. The highest CaO concentration occurred on 200 and 250 mOsm SW (Fig. 1A). However, these tissues differed markedly from those cultured on all other SW concentrations. CaO content in tissues grown on 100, 150, 300 and 350 mOsm was intermediate between that on 0 or 50 mOsm and 200–250 mOsm (Fig. 1A).

Hydration value

There were no differences between hydration values of tissue on 0, 50, 100 and 150 mOsm, 50 and 100 mOsm and those on 250, 300 and 350 mOsm, and 250 and 300 mOsm (Fig. 1A). The differences between tissues on 200 mOsm and 0, 50, 150, 250, 300 and 350 mOsm were more than one standard deviation.

Chlorophyll

In tissues cultured on medium which did not contain SW, chlorophyll concentration was within the range recorded for petioles of greenhouse-grown plants (Table 2). The level of chlorophyll increased gradually in tissues from 0

Table 1. Linsmaier-Skoog medium as used for culture of Colocasia esculenta *var* antiquorum[1]

Component	Amount per liter of culture medium (final concentration in culture medium)	Stock solution (a concentrate prepared for repeated and convenient use)	Volume of stock solution per liter of culture medium	Remarks
Macroelements				
Ammonium nitrate, NH_4NO_3*	1.65 g	82.5 g/l	20 ml	Or weigh
Potassium nitrate, KNO_3*	1.9 g	95 g/l	20 ml	Or weigh
Calcium chloride, $CaCl_2 \cdot 2H_2O$	440 mg	44 g/l	10 ml	
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	370 mg	18.5 g/l	20 ml	
Potassium phosphate, KH_2PO_4	170 mg	8.5 g/l	20 ml	
Chelated iron				
Na_2EDTA	37.3 mg	3.73 g/l	10 ml	One solution
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	27.8 mg	2.78 g/l		
Microelement				
Boric acid, H_3BO_3	6.2 mg	620 mg/l		Add all
Manganese sulfate, $MnSO_4 \cdot 4H_2O$	22.3 mg	2.23 g/l		microelements
Zinc sulfate, $ZnSO_4 \cdot 7H_2O$	8.6 mg	860 mg/l		to the same
Potassium iodide, KI	0.83 mg	83 mg/l	10 ml	1 l,
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.25 mg	25 mg/l		stir and/or
Copper sulfate, $CuSO_4 \cdot 5H_2O$	0.025 mg	2.5 mg/l		heat until
Cobalt chloride $CoCl_2 \cdot 6H_2O$	0.025 mg	2.5 mg/l		dissolved
Polyol				
myo-Inositol	100 mg	No stock	No stock	Weigh
Auxin				
Naphthaleneacetic acid	0.1 mg	100 mg/100 ml 70% ethanol	0.1 ml	Refrigerate stock solution
Cytokinin†				
SD8339 [adenine *N*-benzyl-9-(tetrahydro-2H-pyran-2-yl)]	1 mg	10 mg/10 ml 70% ethanol	1 ml	Refrigerate stock solution
or				
246-Dimethyl amino purine	1 mg	10 mg/10 ml		Stock solution
1 ml (6-DMAP)	Refrigerate	70% ethanol		
Vitamin				
Thiamine HCl (vitamin B_1)	0.4 mg	80 mg/100 ml 70% ethanol	0.5 ml	Refrigerate stock solution
Sugar				
Sucrose	30 g	No stock	No stock	Weigh
Water, distilled‡	To 1000 ml			
Solidifier				
Agar	10 g	No stock	No stock	Weigh

* Solutions containing nitrate and ammonium tend to become contaminated. Therefore, it is preferable not to make stock solutions. If prepared, they must be kept frozen.

† SD8339 is an experimental cytokinin which as of this writing is no longer available and our supply is exhausted. It is listed here because of its use in the original research. Our current work indicates that 6-DMAP is a satisfactory substitute and available. If SD8339 becomes available again in the future one or the other, not both, should be used. The stock solution should be refrigerated.

‡ Adjust pH to 5.6.

Table 2. Calcium oxalate, protein, chlorophyll and alkaloid concentration in parent plants and seedlings of taro UCI Runner[31]

Plants	Calcium oxalate (mg/g fw)	Protein (%)	Chlorophyll (mg/g fw)	Alkaloids, Klett units			
				Secondary, tertiary and amine oxides		Quanternary	
				Mayer's reagent	Wagner's reagent	Mayer's reagent	Wagner's reagent
Corms							
Parents							
Range	5.6–5.9	4.2–5.2		27–24	15–20	31–40	21–40
Average	5.7	4.8		25	18	37	33
Seedlings							
Range	12.0–4.1	6.7–3.8		58–15	31–9	110–27	69–8
Average	7.0	5.0		28	18	50	27
Leaf blades							
Parents							
Range	3.4–3.7			58–110	54–27	179–69	150–58
Average	3.5			89	44	142	116
Seedlings							
Range	5.6–0.5			127–48	80–21	149–58	121–38
Average	2.6			83	43	100	84
Petioles							
Parents							
Tip							
Range			0.4				
Average			0.4				
Middle							
Range			0.3–0.4				
Average			0.4				
Seedlings							
Tip							
Range			1.6–0.3				
Average			0.7				
Middle							
Range			12.0–0.2				
Average			0.5				

to 100 mOsm SW, reached a peak in tissues grown on 150 mOsm, dropped considerably on 200–300 mOsm and decreased further on 350 mOsm (Fig. 1B).

Protein

The protein content (nitrogen level × 6.25) in tissues cultured on 0–150 mOsm and 250–350 mOsm SW was lower than that in corms of mature plants (Fig. 1B). Tissues grown on 200 mOsm had more protein than greenhouse-grown taro corms (Table 2). Protein levels decreased (relative to 0 mOsm) in tissues grown on 50 mOsm, increased on 100 mOsm, remained unchanged on 150 mOsm, rose rapidly on 200 mOsm, and dropped on 250–350 mOsm.

Alkaloid content

Measurements with Mayer's reagent (SAMR) showed that corms of mature greenhouse-grown plants generally contained a lower concentration of secondary alkaloids than cultured tissues

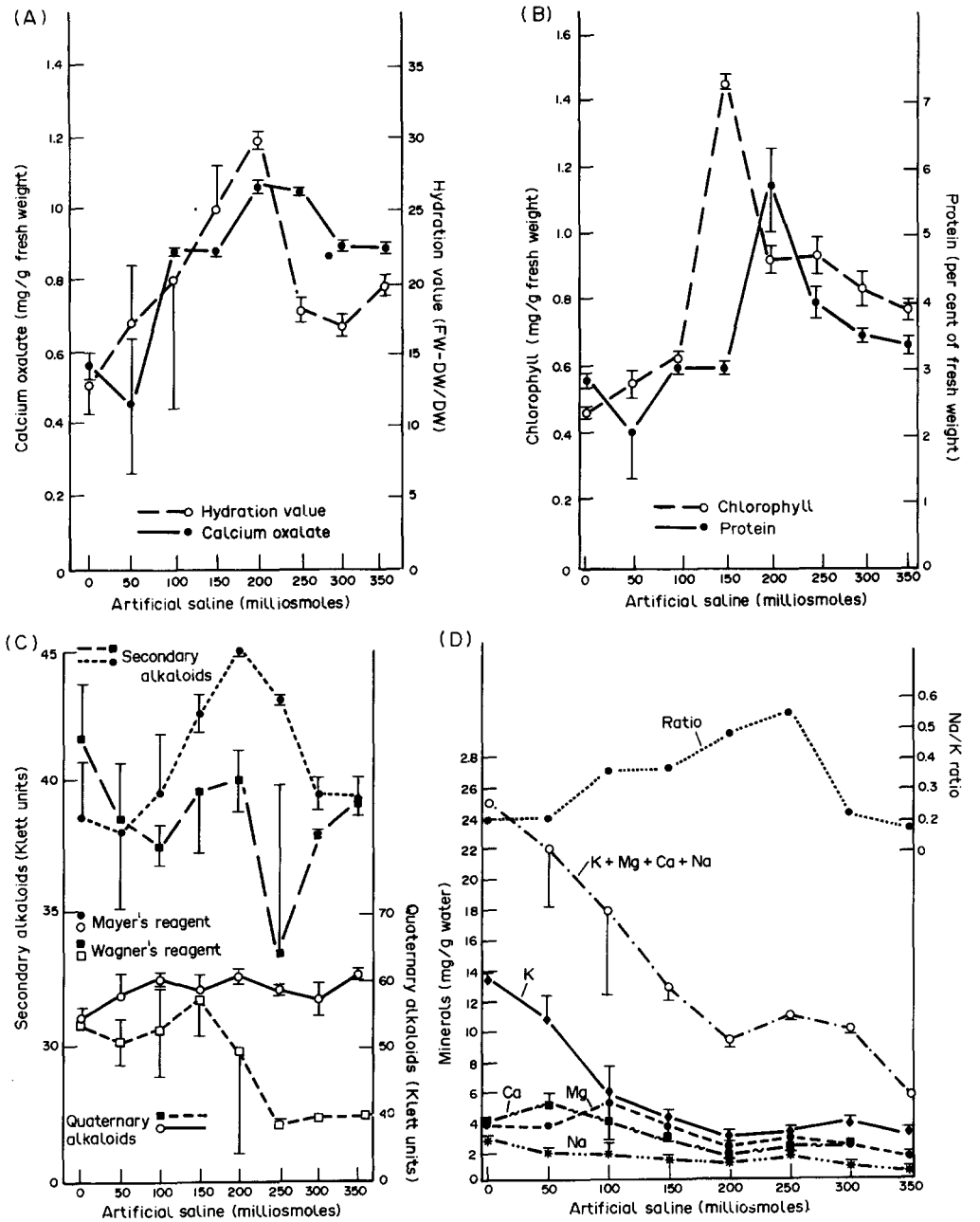

FIG. 1. Constituents of salt tolerant taro. A, Calcium oxalate content and hydration value. B, Protein and chlorophyll concentrations. C, Alkaloid levels. D, Potassium, magnesium, calcium, and sodium concentrations and Na^+/K^+ ratio. Vertical bars are standard deviations.

(Table 2, Fig. 1C). SAMR content in corms of a seedling population was extremely variable; some seedlings contained three times as much as others (Table 2). Levels of SAMR in leaves of mature plants and seedlings were higher than those of cultured tissues (Table 2, Fig. 1C). SAMR concentrations of tissues grown on 0, 50, 100, 300 and 350 mOsm SW were not different from each other (Fig. 1C). The most dramatic differences in SAMR levels occurred between 150 mOsm and 300 and 350 mOsm (Fig. 1C).

Generally, the levels of secondary alkaloids in corms of parents and seedlings as measured by Wagner's reagent (SAWR) were lower than in cultured tissues except on 250 mOsm SW (Table 2, Fig. 1C). SAWR content in leaf blades of parent and seedling plants was variable. In cultured tissues, SAWR levels were within the range, but lower than the means observed in parents and seedlings (Table 2, Fig. 1C). The only notable difference in SAWR levels in cultured tissues was between 0 and 300 mOsm SW (Fig. 1C).

Quarternary alkaloid content of cultured tissues as measured by Mayer's reagent (QAMR) was higher than in corms of mature plants (Table 2, Fig. 1C). In mature plants (leaves) and in seedlings (leaves and corms), QAMR levels were higher than in cultured tissues (Table 2, Fig. 1C). Differences, if any, between QAMR levels over the entire SW range were small (Fig. 1C).

Levels of quarternary alkaloids in cultured tissues as measured by Wagner's reagent (QAWR) were lower than in leaves and higher than in corms of mature plants, and in the range of seedlings (Table 2, Fig. 1C). The differences between QAWR content of tissues grown on 0–150 mOsm and 250–350 mOsm SW were dramatic (Fig. 1C).

Inorganic constituents

Levels of inorganic ions per unit of water in the tissue (mg ion/g water) generally dropped with increasing SW concentrations (Fig. 1D). Sodium levels remained essentially unchanged from 0 to 100 mOsm SW; at 150 mOsm they were lower than the controls, but no different than in tissues grown on 50, 100, 200 and 250 mOsm. On 300 and 350 mOsm, the sodium concentration was less than that of tissues on standard medium. It was also lower than the levels in all other tissues cultured on SW. Magnesium levels were stable from 0 to 100 mOsm SW, dropped on 150 and 200 [relative to 100 mOsm (Fig. 1D)]. Calcium content did not change from 0 to 100 mOsm (Fig. 1D). Potassium levels dropped considerably from 0 to 100 mOsm SW, continued to decline on 150 and 200 mOsm, increased on 250 and 300 mOsm and on 350 mOsm dropped to concentrations which were similar to those on 9200 mOsm (Fig. 1D). The Na^+/K^+ ratio was the same on 0 and 50 mOsm. It increased after that, reaching a peak on 250 mOsm. On 300 and 350 mOsm the ratio dropped to values which were similar to those on 0 and 50 mOsm (Fig. 1D).

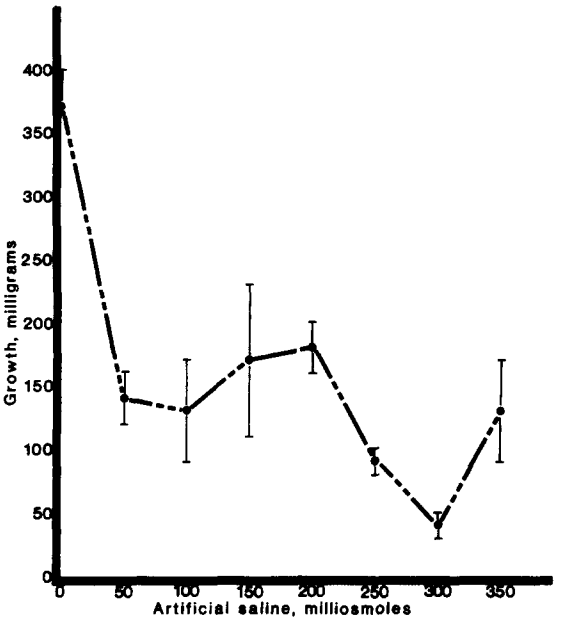

FIG. 2. Cumulative growth in a 4-week period of standard and salt tolerant taro tissues. Each point is the mean of three determinations.

Growth

The initial transfer to SW brought about a substantial decrease in growth of the tissues (Fig. 2). This was followed by a further decrease. Growth on 150 and 200 mOsm increased and afterward dropped to its lowest point before increasing again (Fig. 2).

DISCUSSION

Calcium oxalate

Higher oxalic acid levels were recorded in several halophytes including *Halogenton glomeratus, Bassia, Kochia, Rhagodia* and *Atriplex* species.[7] Several reasons, some suggested in the literature and others by our data, could explain these levels. One suggestion is that CaO may play a role in the ionic balance of tissues.[7,9,10] The fact that CaO content was higher in tissues grown in 100–350 mOsm SW than in those maintained at 0–50 mOsm supports this suggestion. CaO levels may reflect the concentration of oxalic acid in the cells. If this is the case production of oxalic acid essentially doubled between 50 and 100 mOsm SW and increased less dramatically from 150 to 200 mOsm SW. Oxalic acid is toxic at excessively high concentrations. Therefore it is reasonable to assume that it would be restricted to vacuoles[7] where it will precipitate as CaO in the presence of Ca^{2+} due to the low solubility product of CaO:[13] $Ca^{2+} \times C_2O_4^{2-} = 1.8 \times 10^{-9}$ indicates that this must take place. The increased percentage of calcium which is incorporated in CaO when levels of the latter increase suggests that this may indeed be occurring. If so it is possible to speculate that the role played by oxalate and/or CaO in the ionic balance of salt tolerant plants may be of limited importance.

Only a very small proportion of the total calcium is bound as CaO. Such a small proportion probably has negligible effects on the uptake and utilization of calcium ions despite an almost two-fold change in the level of this ion over the range of 0–300 mOsm. The decrease in total calcium from 0 to 200 mOsm SW and the subsequent plateau indicate that uptake and/or release of these ions by the tissues vary with adaptation to salinity.

The levels of CaO were lower in cultured taro tissues than in whole plants. This relationship may occur in other species as well since CaO idioblasts have often been observed to be (1) restricted in distribution, (2)smaller, or (3) nonexistent in callus derived from crystal-containing whole plants.[10,18] Reduced levels of CaO in cultured tissues may result from a number of physiological differences between callus and whole plants,[9,18] or from a reduced availability of calcium *in vitro* relative to the amounts available in soil.[12,27]

Hydration value

The lack of significance and the large standard deviations of HV at 0, 50, 100, 150 and 200 mOsm suggest that the tissues, but not necessarily all individual cells, adapt well to these salinity levels. Adaptation to 250 mOsm is apparently more difficult and this may account for the drop in HV from that on 200 mOsm. The subsequent lack of significant differences and small standard deviation are indicative of a more uniform response by individual cells in the tissue. Except at 200 mOsm, the tissues essentially maintain the same HV over a wide range of SW salinity. This is an indication of effective water content regulation.

Chlorophyll content

In glycophytes exposed to saline conditions, the envelope and internal lamellae of some plastids may be affected adversely.[32] These effects were most severe in cultured taro tissues at SW concentrations above 200 mOsm. The ratio of amyloplasts to chloroplasts in these tissues increased with SW. However, chlorophyll content in tissues cultured in 100–350 mOsm SW was higher than in the controls. The effects of SW on plastid structure and chlorophyll content appear contradictory and this may imply a complex relationship.

Protein content

Amino acids and amines accumulate as a result of exposure to salinity in higher plants.[7] In tomatoes, amino acid nitrogen can increase under salinized conditions from 19 to 82% over control plants.[28] The protein content of seawater tolerant barley can be as high as 12.9%, as opposed to up to 11.5% in fresh water varieties.[6] NaCl salinization, however, causes a decrease in protein accumulation by pea plants.[32] In taro tissues, protein levels remained the same in the range of 0–150 mOsm SW and increased after that. Thus, our results conform only to studies which report increases in protein content with salinization. However, it is also necessary to keep in mind that our protein assay is based on the assumption that a certain proportion of tissue nitrogen is bound in proteins (and that the 6.25 multiplication factor is an accurate reflection of this). In tissues grown

under saline conditions this proportion may vary due to the presence of more free amino acids. Also it is not clear at this point whether salinity can affect nitrogen availability and/or uptake, and if the taro tissues produce excessive amounts of a specific protein and/or amino acid (such as proline, for example).

Alkaloids

Horseradish, corn, peas and other plants accumulate alkaloids and amines under conditions of salinity and/or high NaCl levels.[32] Our finding with Mayer's but not Wagner's reagent, seems to confirm these reports. These reagents are non-specific and their sensitivities for different alkaloids may vary.[8] However, they are very useful for preliminary determinations[29] and general alkaloid assays in plants.[16] Their use in work with taro suggests that *Colocasia* plants may contain a number of structurally different alkaloids.[31] If so, it seems reasonable to assume that the effects of SW on their concentrations may differ. Callus age, media components, and endogenous factors in clones isolated through tissue culture affect the alkaloid content in *Coptis japonica* var *japonica*[36] These factors may have had similar effects on our cultures.

Inorganic constituents

A number of reports are concerned with the ability of glycophytic species to withstand saline conditions. In many instances, these conditions were generated by the addition of NaCl.[4,5,14,15,19,21,33–35] Natural or artificial seawater was used only in a few experiments.[3,4,27,28] Attempts have also been made to select salt tolerant strains of a number of glycophytes. Of particular relevance to our work is the selection of NaCl tolerant alfalfa cells.[5] The concentrations of NaCl employed in the selection of alfalfa cells are roughly the same as those in 200 and 350 mOsm SW. However comparisons between the alfalfa cells and taro tissues must be made with caution due to the presence of other salts in SW and differences between the two plants. Still it is interesting to note that on a dry weight (DW) basis: (1) Na^+ content in taro is generally higher than in alfalfa except in 300 and 350 mOsm, (2) the Na^+ content in taro remains relatively stable between 0 and 250 mOsm SW whereas in alfalfa there is a marked increase, (3) K^+ levels decrease in both plants, but concentrations of this ion in taro are five–six times higher than in alfalfa, (4) the Na^+/K^+ ratio in both plants increases up to 0.75% Na^+ and 250 mOsm SW (which are roughly equivalent) and then stabilizes in alfalfa and drops in taro. The comparison (above) between Na^+ and K^+ content are on a DW basis to allow for comparisons with published data on alfalfa.[5] However, ions are actually dissolved in water and the osmotically significant concentrations are the molarities (Fig. 2).

A common plant response to salinity is increased succulence,[30] which may be accompanied by cell enlargement, and is characterized by increased water content which prevents excessive accumulation of salts in the cell sap.[5] Our results and those obtained with alfalfa[5] tend not to confirm the latter because water content does not increase dramatically. However, large increases in water content may not be necessary in taro tissues since total ion content is inversely related to salinity.

The Na^+/K^+ ratio increased with increasing salinity, reaching a peak at 250 mOsm SW. This is probably due to the fact that sodium content remained relatively stable on 0–250 mOsm SW whereas potassium levels dropped precipitously (Fig. 2). In alfalfa the Na^+/K^+ ratio shows a similar increase before reaching a plateau.[5] These differences in Na^+ and K^+ content may be due to salinity and sulfate ion effects on uptake.[4] In alfalfa, for example, salt selected cells accumulated more K^+ than standard lines.[5] The capacity to maintain high levels of potassium under saline conditions[5] and to utilize this ion in the regulation of sodium may be correlated with salt tolerance in halophytes.

Differences between the ion levels in standard and salt tolerant taro tissues may also be the result of transport systems which have been altered by the selection process. The affinities for and transport of ions may differ.[5] Therefore, comparisons between different plants, tissues, organs, and cell lines require caution.

Growth

The drastic reduction in growth following transfer to 50 mOsm, the subsequent increase and

the decrease after that might be explained by two factors. One possibility is that the initial transfer resulted in the killing of all cells which were sentitive to salinity. A second is that the subsequent increase (100, 150, 200 mOsm) could be due to good growth of cells and tissues which have been selected for a certain degree of salinity tolerance. The same, but at a higher salinity threshold, could have happened in the 200, 250, 300 and 350 mOsm range. These data suggest that during the initial 4 weeks the tissues are adapting to the saline medium, but have not yet become fully tolerant to it.

It is interesting to note that the general shape of the growth curve is paralled by the curves for CaO_x, hydration value, protein level, chlorophyll content, mineral concentration (especially $K^+ + Mg^{2+} + Ca^{2+} + Na^+$ and K^+ alone) and some of the alkaloids. This is not surprising since the levels of ions and/or other constituents may reflect or be a function of growth.

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