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Authors
Nyman, LP
Arditti, J
Bradley, TJ

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

L. P. NYMAN,* J. ARDITTI and T. J. BRADLEY
Department of Developmental and Cell Biology, University of California, Irvine, CA 92717, U.S.A.

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INTRODUCTION

Halophytes and salt tolerant cultivars of several crop plants have been studied extensively in recent years. 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. These components include organic acids, nitrogenous compounds, carbohydrates and mineral ions.

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.

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 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, were obtained by...
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 8359, Shell Research, Modesto, CA) or 1 mg/l dimethylaminopurine (DMAP) and 0.1 mg/l naphthaleneacetic acid (NAA).

Cultures were maintained at 26 ± 1°C under 18 hr photoperiods and light intensity of 2.5 mW/cm².

**Salinity**

To select for salinity tolerance, tissue sections ca 0.1 cm³ were transferred to successively higher concentrations (5%, or 50 mOsm increments) of saline water (SW) approximately every 2.5 months. 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 MgSO₄·7H₂O and 3.80 g MgCl₂ in 100 ml of double distilled water. 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. 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. 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. Alkaloid concentrations were measured with Wagner’s and Mayer’s reagents. This technique was modified only to the extent that a Klett colorimeter was used to measure turbidity of thoroughly agitated samples.

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

**Inorganic analyses**

Tissues were dried at 50 ± 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. 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

| 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, NH₄NO₃*   | 1.65 g                                                                     | 82.5 g/l                                                              | 20 ml                                                | Or weigh |
| Potassium nitrate, KNO₃*    | 1.9 g                                                                       | 95 g/l                                                                | 20 ml                                                | Or weigh |
| Calcium chloride, CaCl₂·2H₂O| 440 mg                                                                      | 44 g/l                                                                | 10 ml                                                |         |
| Magnesium sulfate, MgSO₄·7H₂O| 370 mg                                                                     | 18.5 g/l                                                              | 20 ml                                                |         |
| Potassium phosphate, KH₂PO₄| 170 mg                                                                      | 8.5 g/l                                                                | 20 ml                                                |         |
| **Chelated iron**           |                                                                             |                                                                        |                                                      |         |
| Na₂EDTA                    | 37.3 mg                                                                     | 3.73 g/l                                                              | 10 ml                                                | One solution |
| Ferrous sulfate, FeSO₄·7H₂O | 27.8 mg                                                                     | 2.78 g/l                                                              |                                                      |         |
| **Microelement**            |                                                                             |                                                                        |                                                      |         |
| Boric acid, H₃BO₃           | 6.2 mg                                                                      | 620 mg/l                                                              |                                                      | Add all |
| Manganese sulfate, MnSO₄·4H₂O| 22.3 mg                                                                     | 2.23 g/l                                                              |                                                      | microelements |
| Zinc sulfate, ZnSO₄·7H₂O    | 8.6 mg                                                                      | 860 mg/l                                                              |                                                      | to the same |
| Potassium iodide, KI        | 0.83 mg                                                                     | 83 mg/l                                                               | 1 l                                                  |         |
| Sodium molybdate, Na₂MoO₄·2H₂O| 0.25 mg                                                                     | 25 mg/l                                                               | 10 ml                                                | stir and/or |
| Copper sulfate, CuSO₄·5H₂O  | 0.025 mg                                                                    | 2.5 mg/l                                                              |                                                      | heat until |
| Cobalt chloride CoCl₂·6H₂O  | 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                                                         | 0.1 ml                                               | Refrigerate stock solution |
| **Cytokinin†**              |                                                                             |                                                                        |                                                      |         |
| SD8339 [adenine N-benzyl-9- (tetrahydro-2H-pyran-2-yl)]| 1 mg                              | 10 mg/10 ml                                                          | 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 B₁)   | 0.4 mg                                                                      | 80 mg/100 ml                                                          | 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

| Plants          | Calcium oxalate (mg/g fw) | Protein (%) | Chlorophyll (mg/g fw) | Mayer's reagent | Wagner's reagent | Alkaloids, Klett units |
|-----------------|---------------------------|-------------|-----------------------|----------------|----------------|------------------------|
|                 |                           |             |                       | Secondary, tertiary and amine oxides | Quaternary   |
| Corms Parents   |                           |             |                       | Mayer's reagent | Wagner's reagent | Mayer's reagent        | Wagner's reagent |
| 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 | 1.6–0.3 | 0.7 | 1.6–0.3 |
| Average | 0.4 | 0.7 | 0.5 | 0.5 |
| Middle Range | 0.3–0.4 | 12.0–0.2 | |
| Average | 12.0–0.2 | 0.5 | |

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.
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).

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).
Calcium oxalate

Higher oxalic acid levels were recorded in several halophytes including Halogenton glomeratus, Bassia, Kochia, Rhagodia and Atriplex species. 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. 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 where it will precipitate as CaO in the presence of Ca²⁺ due to the low solubility product of CaO₂⁻: Ca²⁺ × C₂O₄⁻ = 1.8 × 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) non-existent in callus derived from crystal-containing whole plants. Reduced levels of CaO in cultured tissues may result from a number of physiological differences between callus and whole plants, or from a reduced availability of calcium in vitro relative to the amounts available in soil.

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. 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. In tomatoes, amino acid nitrogen can increase under salinized conditions from 19 to 82% over control plants. 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. NaCl salinization, however, causes a decrease in protein accumulation by pea plants. 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 tao 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. Our finding with Mayer’s but not Wagner’s reagent, seems to confirm these reports. These reagents are finding with Mayer’s but not Wagner’s reagent, conditions of salinity and/or high saline conditions. In many instances, these con­ditions were generated by the addition of NaCl salts in SW, (which are roughly equivalent) and then stabilizes in alfalfa and drops in tao. The comparison (above) between Na+ and K+ content are on a DW basis to allow for comparisons with published data on alfalfa. 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, which may be accompanied by cell enlargement, and is characterized by increased water content which prevents excessive accumulation of salts in the cell sap. Our results and those obtained with alfalfa tend not to confirm the latter because water content does not increase dramatically. However, large increases in water content may not be necessary in tao 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. These differences in Na+ and K+ content may be due to salinity and sulfate ion effects on uptake. In alfalfa, for example, salt selected cells accumulated more K+ than standard lines. The capacity to maintain high levels of potassium under saline conditions 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 tao 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. 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 sensitive 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 paralleled by the curves for CaO₂, hydration value, protein level, chlorophyll content, mineral concentration (especially K⁺ + Mg²⁺ + Ca²⁺ + 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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