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EFFECTS OF ARTIFICIAL SEAWATER ON THE ULTRASTRUCTURE AND MORPHOMETRY OF TARO (*COLOCASIA ESCULENTA*, ARACEAE) CELLS *IN VITRO**

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NYMAN L. P., WALTER R. J., DONOVAN R. D., BERNS M. W. and ARDITTI J. *Effects of artificial seawater on the ultrastructure and morphometry of taro (Colocasia esculenta, Araceae) cells in vitro*. ENVIRONMENTAL AND EXPERIMENTAL BOTANY 27, 245-252, 1987.—Cultured tissues of taro, *Colocasia esculenta* var *antiquorum*, were grown on modified Linsmaier-Skoog medium with and without the addition of 50–350 mOs (5–35%) artificial seawater (ASW). Cell wall thickness and cross-sectional area were variable, but not significantly different over the range of ASW concentrations from 0 to 300 mOs. However, cells at 350 mOs ASW did have significantly thinner walls and smaller cross-sectional areas. Of the organelles observed, plastids appeared to be the most sensitive to increasing ASW concentrations. There were more plastids per unit area at 350 mOs ASW than at any other concentration, but their size was reduced. The ratio of amyloplasts to chloroplasts increased with increasing ASW levels. Mitochondrial and nuclear membranes did not seem to be affected by the ASW concentrations used. At high ASW levels, nucleoli showed a loss of electron density in their central regions.

INTRODUCTION

MUCH OF the information on the structure, ultrastructure and morphometry of salt tolerant plants has been obtained from investigations on the effects of high concentrations of NaCl on whole plants and/or tissues *in vitro*.^(7,9,12,13,15,17,18) Few experiments have been conducted to determine the effects of a graded series of NaCl or seawater on plant morphology and anatomy.^(8,13) A relatively small number of studies have been carried out with seawater (artificial or natural) on these features.

As part of our program to develop and characterize salt tolerant taro,⁽¹¹⁾ we have studied (1) chemical constituents⁽¹¹⁾ and (2) structural fea-

tures⁽²⁰⁾ of tissues cultured on media containing several concentrations of artificial seawater. Growth on such media can be expected to more nearly approximate cultivation on seawater containing soils than NaCl alone.

MATERIALS AND METHODS

Tissue culture

Tissues of *Colocasia esculenta* var *antiquorum*, UCI Runner were cultured in modified liquid Linsmaier-Skoog (LS) medium^(2,10) containing 0–350 milliosmoles (mOs; 0–35%) artificial seawater (ASW).⁽⁴⁾ All cultures were grown in the presence of 1 mg l⁻¹ 6-dimethylaminopurine (DMAP,

* This paper is dedicated to Dr Peter van Schaik, formerly of USDA, for his continuous support of taro research.

Sigma Chemical Co., St. Louis, MO) and 0.1 mg l^{-1} naphthaleneacetic acid (NAA, Sigma Chemical Co.). Saline media were prepared by adding to LS appropriate volumes of ASW⁽⁴⁾ stock solution.

Cultures were maintained at $25 \pm 2^\circ\text{C}$ and illuminated with Gro Lux and incandescent lamps at a light intensity of 4.4 mW cm^{-2} , and 18 hr photoperiods. Tissues were subcultured every 2–4 months. Liquid cultures were initiated on a rotary shaker at 1 rpm. They were transferred to a reciprocal shaker (travel distance 10 cm and 29 oscillations min^{-1}) following proliferation.

Microscopy

Samples of tissues that grew well (i.e. green, rapidly proliferating tissues which had no necrotic spots) at each ASW level were selected for observations. Tissues for transmission electron microscopy (TEM) were cut into pieces no larger than 0.25 cm^3 , fixed 12 hr in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and postfixed in 2% aqueous osmium in 0.1 M sodium cacodylate buffer at 0°C overnight. They were then washed and dehydrated in acetone at 0°C , brought to room temperature, infiltrated with Spurr's resin and embedded for 18 hr at 70°C . This procedure was selected on the basis of preliminary experiments (unpublished). Silver sections were cut with an ultra-microtome, double stained with 10% uranyl acetate in absolute methanol and Reynold's lead citrate solution, and observed with a JEOL JEM 100C electron microscope.

To stabilize tissues taken from samples grown in ASW media, sucrose was added to the fixation solution as an osmoticum. In preliminary experiments (unpublished), 0.63 M sucrose was found to be suitable (tissues did not swell or shrink and were not damaged) to stabilize tissues grown on 350 mOs ASW. Sucrose levels necessary to stabilize tissues grown in the presence of other ASW concentrations were calculated based upon 0.09 M sucrose/50 mOs ASW.

Material for light microscopy was prepared in the same way as samples used for TEM. Serial plastic sections $2 \mu\text{m}$ thick were cut at right angles to the tissue surface, were affixed to glass slides by

heating on a hot plate, and were stained with 0.05% aqueous toluidine blue.

Morphometry

Cell and chloroplast cross sectional areas, cell wall thickness, and numbers of chloroplasts per cell cross sectional area were determined using computer enhanced video microscopy.^(19,20) This method produces a digitally processed light microscope image which can be measured electronically. Measurements were then integrated and analyzed statistically. The tissues were very heterogeneous⁽¹⁰⁾ and of different organ and cell types.⁽¹⁰⁾ To facilitate comparisons between treatments, relatively uniform flat regions of each tissue were chosen for observations. Ten cells 1–5 layers below the outer uniseriate layer were chosen randomly from two separate calloids for each ASW treatment. All chloroplasts in the calloid cells were counted and measured. Cell wall thickness was measured at three randomly chosen points of the walls of each cell. Where possible, at least 50 chloroplasts were measured per ASW concentration.

Analysis of variance (ANOVA)

The data were subjected to one way ANOVA using a program for an Apple II Computer (Anova, Synacomp, Inc., 1427 Monroe Avenue, Rochester, NY 14618). Least significant differences (LSD) were also calculated.⁽¹⁶⁾

RESULTS

Morphometry

At 350 mOs, cell walls are significantly thinner than those of cells grown on 0, 50, 100, 150, 200 and 250 mOs. Cell wall thickness at other ASW levels are not significantly different (Fig. 1A). The number of chloroplasts per $\mu\text{m}^2 \times 10^3$ cell cross-sectional area dropped at 50 mOs, increased on 100 mOs and remained stable on 150 mOs, 200 mOs, 250 mOs and 300 mOs before increasing exponentially at 350 mOs (Fig. 1B). However, the individual chloroplast area/cell cross sectional area ratio on all ASW levels (except 50 and 100 mOs) was similar to that of cells cultured on standard medium (Fig. 1B). Smallest ratios occurred at 50, 100 and 250 mOs ASW (Fig. 1B). Total chloroplast area/cell cross-sectional area ratio was

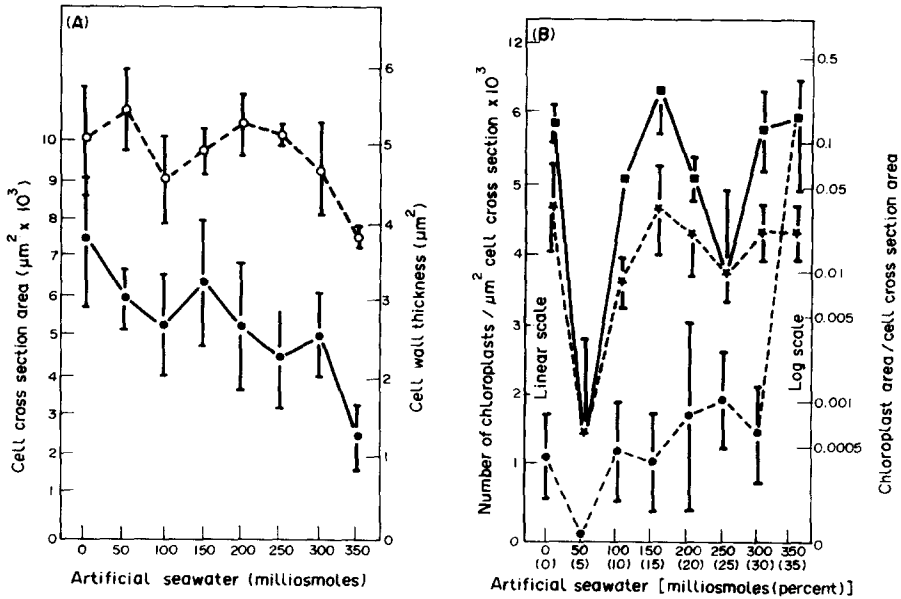


Fig. 1. Effects of artificial seawater on taro cell morphometry. (A) Cell cross sectional area (solid line and closed circle, LSD = 2.6) and cell wall thickness (dotted line and open circles, LSD = 1.06). (B) Number of chloroplasts per μm^2 (dotted line and closed circles), ratio between the average area of individual chloroplasts and cell cross section area (slashes and asterisks, LSD = 0.018) and ratio between total chloroplast area and cell cross section area (solid line and squares, LSD = 0.061). Vertical bars represent standard deviations. LSD is the least significant difference.⁽¹⁶⁾

lowest at 50 and 250 mOs ASW (Fig. 1B). Chloroplast size was generally inversely proportional to ASW level.

Cytology

Plastids in cells grown on standard LS medium without ASW were spherical or crescent-shaped. Almost all plastids present in cells from the outer regions of the tissue were chloroplasts (Fig. 2A). Amyloplasts were also present, occurring in higher proportions in the deeper layers of the ground tissue. Grana lamellae, intergranal thylakoids and the stroma matrix were usually clearly defined in chloroplasts (Fig. 2A). Very small starch grains were occasionally found in these plastids.

The effects of ASW on cells included dramatic changes in (1) plastid shape, (2) the appearance of internal membranes, (3) the ratio of grana to stroma, and (4) the occurrence of internal inclusions within the plastid (Figs. 2B–2F). Few

plastids were present in tissue grown in 50 mOs ASW. Those observed were either linear or round and had irregularities in their outer membranes. Plastids in cells from tissue grown in 100 mOs ASW were more abundant than on 50 mOs, spherical to oval in shape, and contained starch grains as well as lightly-stained spherical inclusions. There were clear disruptions in the outer plastid membrane as well as an apparent breakdown in thylakoid membranes. In cells from tissues grown at 150 mOs ASW, plastids were either irregular in shape (Fig. 2B), or nearly crescent-shaped (Fig. 2C) and tended to be relatively large compared to those observed at 0–100 mOs ASW. As with the 100 mOs treatments, starch grains as well as other lightly-stained inclusions were observed in these plastids.

Plastids in cells grown in 200–350 mOs ASW generally had extremely reduced internal membrane systems and stroma (Figs. 2D–2F). At 200 and 250 mOs ASW, plastids tended to be small,

elongated or spherical structures which contained either single large starch grains or a number of small grains (Fig. 2D). Plastids were generally present in all cells with the exception of those that differentiated as tannin or crystal idioblasts.

Ultrastructural observations revealed that the general morphology of nuclei was unaffected by increased ASW concentrations (Figs. 2G, 2H). The nuclear membrane was intact in all cells and nuclei were spherical to lobed. Condensation of chromatin was similar in nuclei from all ASW concentrations. Nucleoli in cells of calloid grown on ASW free LS medium were spherical evenly stained bodies (Fig. 2G). Only one nucleolus was observed in each nucleus. In the higher concentrations of ASW, however, nucleoli were less electron dense in their central regions (Fig. 2H).

Mitochondria in cells from all treatments could not be readily distinguished. Some large and lobed mitochondria were observed, but most appeared to be spherical to oval in shape (Figs. 2A, 2D, 2F). No gross abnormalities were noted in the appearance of the cristae or outer double membrane.

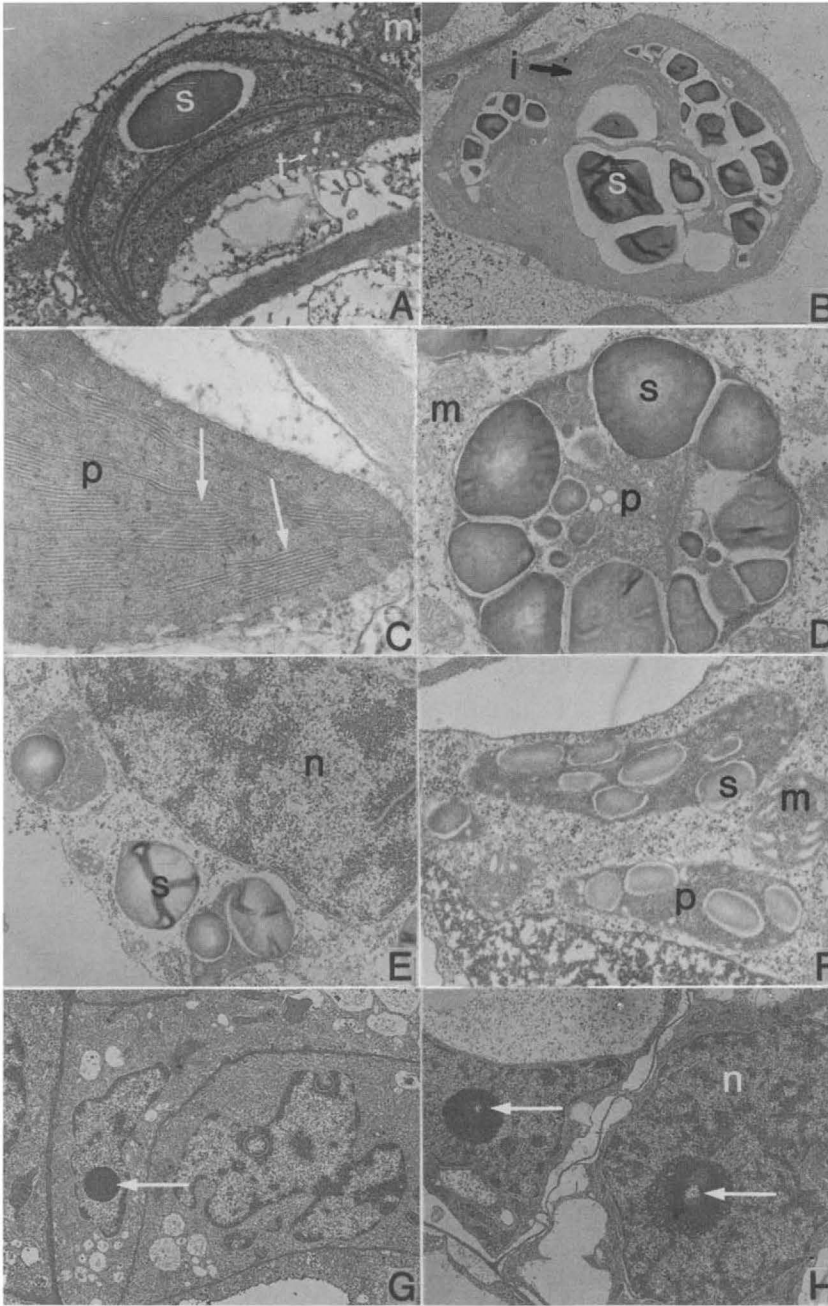
DISCUSSION

The available literature on ultrastructural changes associated with increased salinity is concerned primarily with NaCl and its toxic

effects.^(6,17) This salt is the major component of seawater and, therefore, it is not surprising that ASW effects appear to be strikingly similar to those caused by NaCl. Also, some studies which examine effects of stress on plant ultrastructure have failed to balance the osmotic potentials of fixatives and tissues (for a review see 17). Such imbalances can result in damage to cellular components. Our results show that this problem can be eliminated by adjusting the osmolarity of the fixatives.

Reduction in cell size at 350 mOs ASW may in part reflect a block in the utilization of reserve material, since it was accompanied by an increase in starch and electron dense inclusions, which most probably were lipid droplets. Increased storage of starch and lipid in plastids exposed to NaCl may reflect an initial increase in their production followed by a block in utilization.⁽¹⁷⁾ The fact that taro plastids became amyloplast-like with exposure to ASW supports this view. Cell area may also be a function of changes in endogenous hormone levels. Both abscisic acid and cytokinin levels can be directly affected by salinity.⁽¹⁴⁾ However, adaptation of taro cells to 50–300 mOs ASW was not associated with significant changes in cell size or wall thickness. Therefore it seems unlikely that salinity tolerance (at least at the lower ASW levels) is a direct result of modifications in growth regulator concentration.

FIG. 2. Ultrastructure of cells from taro tissues cultured *in vitro*. (A) Plastid from a calloid grown in standard Linsmaier-Skoog medium. The internal grana and stroma matrices are clearly defined and starch grains (s) are present. Several very small electron transparent regions (t) are visible within the stroma. Most plastids in the outer 5–6 layers of these calloid cells are crescent-shaped. Mitochondria (m) are spherical to oval in outline and have well-defined cristae. $\times 9600$. (B) Plastid from a calloid cell grown on Linsmaier-Skoog medium containing 150 mOs artificial seawater. Starch grains (s) and spherical inclusions (i) are present in the stroma of plastids. $\times 9600$. (C) Part of a crescent-shaped plastid (p) from a calloid grown in 150 mOs artificial seawater. Grana membranes (arrows) are visible and stroma is very electron dense. $\times 20,800$. (D) Cell from a calloid grown in 250 mOs. Numerous mitochondria (m) are visible in the cytoplasm. Plastids (p) have very reduced stroma-matrices and relatively large starch grains (s). $\times 8000$. (E) Plastids (p) and nucleus (n) from a calloid cell grown in 300 mOs. Starch grains (s) are visible in the stroma. $\times 5280$. (F) Calloid grown in 350 mOs artificial seawater. The mitochondria (m) have visible cristae and are spherical to elliptical in outline. Plastids consist mostly of starch grains (s) with very reduced stroma matrices. $\times 16,000$. (G) Cells from a meristematic region of a calloid grown on standard Linsmaier-Skoog medium. The nucleoli (arrow) are round and electron dense. $\times 1920$. (H) Cells from the meristematic region of a calloid grown on 350 mOs. The nuclei have a similar range of shapes to those observed in medium without artificial seawater (Fig. 2G), and the appearance of the nucleoplasm (n) is also similar. Nucleoli have electron transparent outer regions both near their surface and in a roughly central position (arrows). $\times 20,800$.



Cell and chloroplast size are very sensitive to changes in the ionic composition of their surroundings and undergo changes in (1) size, (2) membrane appearance, (3) starch and lipid accumulation, (4) photosynthetic activity and (5) degree of vacuolation,^(1,3,7,13,17) in the presence of NaCl. Similar variability in cell and plastid size and cell wall thickness occurred in taro subjected to ASW, but there was a significant reduction in the size of cells, cell wall and plastids at 350 mOs.

In a previous report, mitochondria appeared more stable than other organelles under conditions of increased NaCl but became less electron dense and developed swollen cristae.⁽¹⁷⁾ Further, nuclei were unchanged by increased NaCl concentration except for swelling of the outer double membrane. Other membranes including the tonoplast and plasmalemma appeared disrupted or swollen as well.⁽³⁾ Nuclei, nucleoli and mitochondria in taro tissue exposed to ASW seemed to be more stable than plastids although there was a clarification of the central portion of nucleoli in 350 mOs ASW. Changes in nucleolar appearance are in agreement with reports regarding cells of other species exposed to NaCl,⁽¹⁷⁾ and may reflect alterations in the rate of RNA synthesis.⁽⁵⁾ In the presence of NaCl, nucleoli often became lobed or irregular in shape. Two nucleoli were observed in some cells exposed to NaCl, possibly reflecting an increase in ploidy level.⁽¹⁷⁾

Altogether organelles of taro tissue exposed to ASW were relatively stable, plastids being the only exception. The reason for these differences is unclear at present.⁽¹⁷⁾

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