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# EFFECTS OF ARTIFICIAL SEAWATER ON MORPHOLOGY AND ANATOMY OF TARO (*COLOCASIA ESCULENTA* VAR *ANTIQUORUM*, ARACEAE) TISSUES *IN VITRO*

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*Biological Sciences, California State Polytechnic University, 3801 West Temple Ave., Pomona, CA 91768, U.S.A. and †Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA 92717, U.S.A.

NYMAN L. P. and ARDITTI J. A. *Effects of artificial seawater on morphology and anatomy of taro* (Colocasia esculenta *var* antiquorum, *Araceae*) *tissues* in vitro. ENVIRONMENTAL AND EXPERIMENTAL BOTANY **28**, 215–224, 1988. Taro tissues were cultured in 50–500 milliosmoles (mOs) artificial seawater (ASW). Only those in 50–350 were stable and survived prolonged culture. Organogenesis of shoots, leaves and leaf-like structures was similar in all ASW concentrations, although more abnormal growth was observed at 300–350 mOs. All cultures had highly organized tissues including procambium or vascular tissue, tannin-containing idioblasts and vacuolate parenchyma cells. Differentiation of a recognizable epidermis with stomata was most pronounced in liquid culture.

## INTRODUCTION

TARO tissues cultured on standard media undergo reversible structural changes from a slow-growing callus to a teratoma-like mass we have termed calloid.[9] Most of the calloid growth for the first 6–9 months of culture was tumor-like.[9] When the calloids were cultured on a graded series of artificial seawater (ASW) concentrations it was possible to select for salt tolerant tissues which produced plantlets. However, the number and quality of plantlets produced per calloid culture varied with ASW concentration, hormone balance and age of the tissue.[8,9] Cultures in the middle concentrations of ASW (150–250) produced higher numbers of plantlets which appeared normal.[7] Plantlets produced *in vitro* had stunted shoots, under-developed root systems and survived in soil for limited periods. This study was undertaken to obtain more information about the effects of increasing salinity on (1) shoot morphogenesis and leaf development, (2) tissue

differentiation within the calloid, and (3) production of tumor-like structures.

## MATERIALS AND METHODS

*Culture methods*

Tissues were cultured on Linsmaier–Skoog (LS) medium[1] containing 1 mg$^{-1}$ 6-dimethylaminopurine (6-DMAP) and 0.1 mg$^{-1}$ naphthaleneacetic acid (NAA). Cultures were maintained at $25 \pm 2°C$, illuminated with Gro Lux and incandescent lamps at a light intensity of 4.4 mW cm$^{-2}$ and given 18 hr photoperiods. Subcultures were made every 2–4 months. Liquid cultures were initiated on a rotary shaker at 1 rpm and transferred to a reciprocal one at 29 oscillations min$^{-1}$.

The media containing ASW were prepared as described previously.[2,8,9] To select for salt tolerance, tissue was transferred from standard LS medium to successively higher levels of ASW at

50 milliosmole (mOs) increments. Transfers to higher concentrations of ASW were made only after the tissues became established and grew well. The highest concentration of ASW in which tissues remained stable was 350 mOs.

*Microscopy*

Plant material for light microscopy was cut into segments no larger than 0.25 cm$^2$, fixed overnight in 4% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2), washed and dehydrated in acetone at 0°C. Tissue was gradually brought to room temperature, infiltrated with Spurr resin and embedded at 70°C for 18 hr. Sections, 2 $\mu$m thick, were cut with a JB-4 Sorvall Porter Blum microtome and stained in 0.1% aqueous toluidine blue.

For SEM observations tissue was sectioned into 1 mm$^3$ segments and fixed for 14–18 hr in 4% glutaraldehyde buffered with sodium cacodylate at pH 7.2. After a slow ethanol dehydration (10% increments of ethanol for 20–30 min at each concentration) and a similarly slow infiltration with freon, the tissue was dried in a Bomar 900 EX critical point dryer. The sections were then mounted and coated with gold for 3 min in a Hummer 2 Sputter coater. Observations were made in a Hitachi S-500 scanning electron microscope.

To stabilize tissues taken from samples grown in seawater-containing media, sucrose was added to the fixation solution. A concentration of 0.63 M sucrose was empirically found suitable for tissues grown on 350 mOs ASW. Sucrose concentrations necessary to stabilize tissues from other levels of ASW were calculated mathematically (i.e. 0.09 M sucrose for 50 mOs ASW, 0.18 M sucrose for 100 mOs ASW, 0.27 M sucrose for 150 mOs ASW, etc.)

## RESULTS

*Effects of ASW on calloid and plantlets*

Healthy green calloids were established in ASW concentrations of 50–350 mOs. No differences in gross morphology were noted in young (4–8 weeks) calloid cultures placed on 0 or 50–350 mOs ASW. Tissues survived for a brief period on 400–500 mOs ASW, but necrosis usually developed after about 1 month of culture.

Plantlets generated on all ASW concentrations varied in (1) overall size, (2) leaf dimension and shape, (3) extent of internodal elongation, (4) degree of basal shoot formation, and (5) root development. These differences were evident in plantlets of similar ages produced synchronously in the same culture vessel. After transfer to indi-

FIG. 1. Scanning electron microscope images of calloids from standard and artificial seawater-containing cultures. (a) Primordium (p) associated with a meristemoid region (arrows). These structures arose from a calloid growing on standard semi-solid Linsmaier–Skoog medium. Usually the first primordium associated with a meristemoid differentiated as a linear-shaped organ [see Figs 1(b), (c) and 3(d)]. × 136. (b) Linear-shaped organ (L) with stomata (s) grown on standard semi-solid Linsmaier–Skoog medium containing 50 mOs artificial seawater. × 21. (c) Plantlet with linear (L) and spatulate leaf-like structures (sp) grown on liquid Linsmaier–Skoog medium containing 50 mOs artificial seawater. × 21. (d) Leaf with accuminate tip and rounded base (Le) arising from a calloid grown on liquid Linsmaier–Skoog medium containing 50 mOs artificial seawater. The structure in the foreground appears to be a shortened leaf-like organ (o) similar to those borne on plantlets [see Fig 1(c)]. × 21. (e) Meristemoid region on a calloid grown in liquid Linsmaier–Skoog containing 100 mOs artificial seawater on standard liquid Linsmaier–Skoog medium. The tip of a primordium (p) is visible. The sheathing nature of the leaves and leaf-like organs is very marked in this region. × 110. (f) Meristemoid region on a calloid grown on semi-solid Linsmaier-Skoog medium containing 150 mOs artificial seawater. One of the older leaf-like organs was removed so that the young tissue below is visible. A linear organ (L) is visible. × 153.
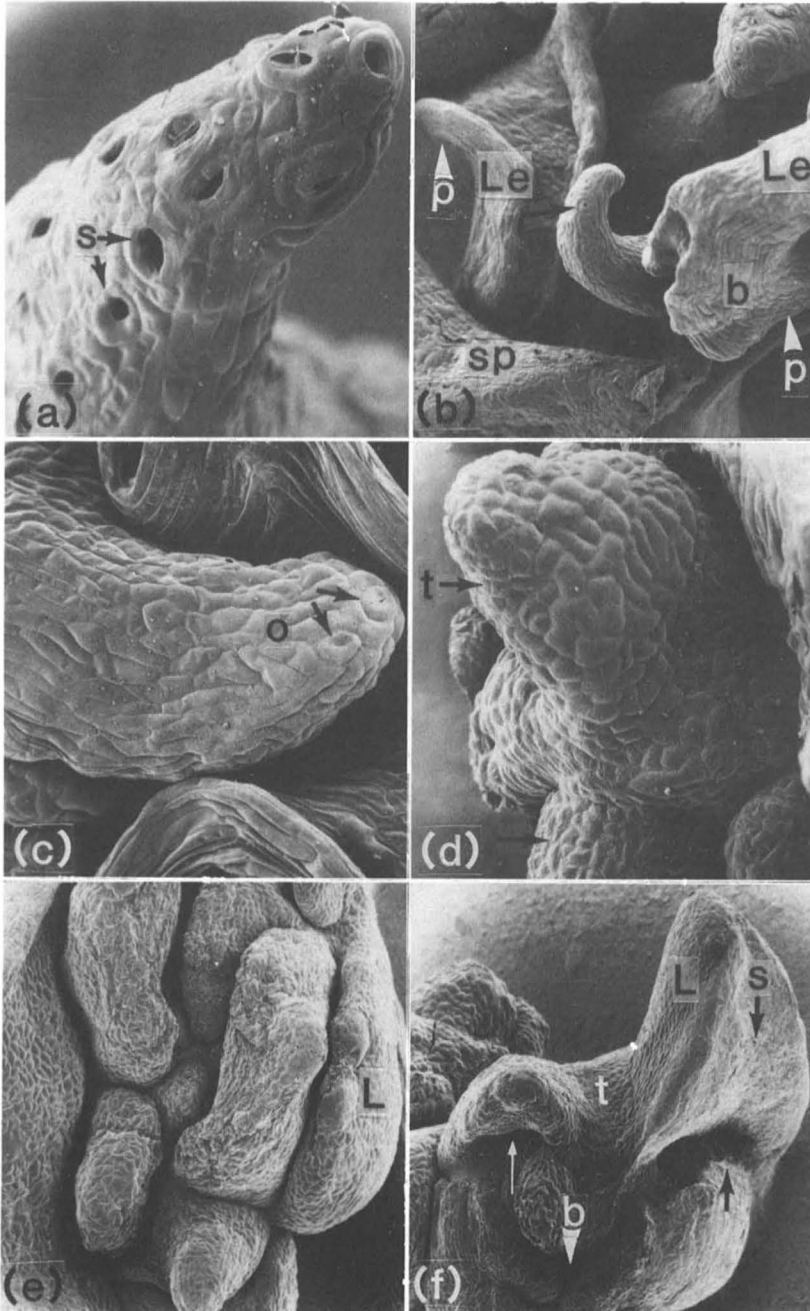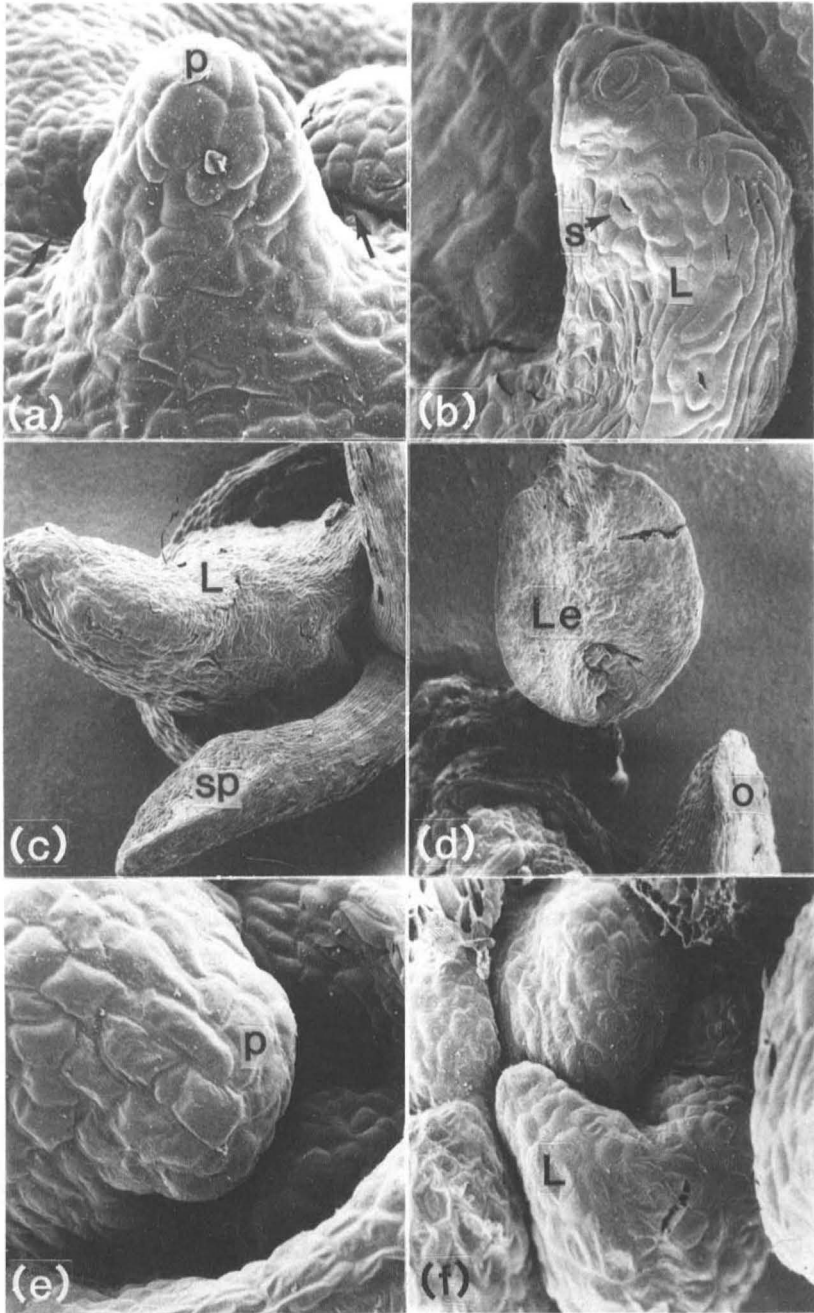
FIG. 1.

FIG. 2.

vidual flasks, differences between plantlets often remained constant.

*Morphological features of ASW calloids*

Observation of 32 week-old calloids under the SEM showed that protocorm-like bodies, multiple meristem regions, abnormal shoot and leaf-like structures as well as normally-appearing plantlets were produced at all levels of ASW tested [Figs 1(a)–(f), 2(a)–(f), 3(a)].

The meristematic regions and the primordia produced from them were structurally variable. Variability seemed to be independent of ASW concentration. Leaf-like primordia which did not differentiate beyond small projections [Fig. 1(a)] or linear elongated structures [Figs 1(b), (c), 2(a)–(c)] arose on certain regions of calloids from all cultures. There was an obvious gradation from small determinate mounds of tissue [Fig. 1(a)] to spatulate projections which were more leaf-like [Fig. 1(c)]. Most meristematic regions produced linear or spatulate organs at the oldest nodes with hastate or peltate leaves developing subsequently [Figs 1(c), 2(b)]. Occasionally nearly peltate leaves with rounded bases and acute tips resembling those of young seedlings [Fig. 1(d)] arose directly from a flat meristem.

Although plants produced from seed generally show complete sheathing of petiole bases around the shoot apex, calloid leaf and leaf-like projections usually [Figs 1(c), 2(b)] but not always [Fig. 1(e)] had incomplete sheath formation. This diversity was most evident in 200 mOs ASW liquid cultures where many meristems in close proximity produced leaf-like structures [Fig. 2(b)].

Most calloid masses had at least some differentiated stomata on regions other than surfaces of normal leaf primordia [Figs 1(b), 2(a), (b), (f), 3(b)]. Numbers of stomata were highest on calloids grown in 200 mOs ASW liquid cultures [Fig. 2(b)] but normal numbers were observed on semi-solid medium at the same ASW level. There were no other consistent differences between liquid and semi-solid cultures in the appearance of cells of the outer calloid layer, over the entire range of ASW concentrations used. The outer cells were either irregular [Fig. 1(a)] or rectangular in shape with their long axes parallel to the elongation plane of the leaf or leaf-like organ upon which they were found [Figs 1(b), 2(a), (c)].

Localized areas with a teratomatous appearance were observed in all cultures [Figs 2(d)–(f)],

FIG. 2. Scanning electron microscope images of calloids from standard and artificial seawater-containing cultures. (a) Linear-shaped structure which arose from a calloid grown on liquid Linsmaier–Skoog medium containing 150 mOs artificial seawater. Stomata (s) were more numerous in liquid than on semi-solid medium. × 170. (b) Calloid grown on liquid Linsmaier–Skoog medium containing 200 mOs artificial seawater. Leaf primordia (Le) of two plantlets (p) are shown illustrating the wide range of morphologies of the leaves and leaf-like organs. Numerous stomata (arrow) are visible on the surfaces of all organs. A spatulate leaf (sp) is shown and the basal sheaths (b) of the petiole are also evident. × 36. (c) Linear-shaped organ (o) which arose from a calloid grown on liquid Linsmaier–Skoog medium containing 200 mOs artificial seawater. Stomata (arrows) can be seen near the tip of the structure. Elongated cells on the surface may be associated with internal vascular tissue. × 153. (d) Tumor-like region (t) from a calloid grown on semi-solid Linsmaier–Skoog medium containing 200 mOs artificial seawater. Spherical protocorm-like structures (arrow) associated with these areas are shown. × 119. (e) Region of a calloid grown on liquid Linsmaier–Skoog medium containing 250 mOs artificial seawater. Many leaf-like primordia (L) are arising in close proximity. × 41. (f) Tumor-like (t) region from a calloid grown on liquid Linsmaier–Skoog medium containing 250 mOs artificial seawater. The leaf-like organs (L) had stomata (s) on their surface and appeared to be arising from two centers of meristematic activity (arrows). The bifurcated appearance of the basal sheath below (b) indicates that this region may have functioned as a single meristemoid initially which branched later in its development. × 29.

but were in common in 300 and 350 mOs ASW [Fig. 3(a)]. These regions had closely appressed round or oblong organs [Fig. 2(d)] or large numbers of leaf-like structures which originated from one or more centers of meristematic activity [Figs 2(e), (f)]. Occasionally stomata were observed in these regions [Fig. 2(f)].

*Anatomical features of ASW calloids*

In section, ground tissues of the calloid from all levels of ASW appeared similar. Parenchyma cells were vacuolate and usually circular to oval in shape as viewed in planes both perpendicular and parallel to the calloid surface [Figs 3(c)–(f), 4(b)–(d)]. Tannin-containing idioblasts were common in the internal layers [Figs 3(f), 4(a), (c) (d)]. Crystal-containing idioblasts were observed infrequently (Nyman, unpublished). Procambium or fully developed vascular tissue was often present below regions of meristematic activity and in association with leaves or leaf-like primordia [Figs 3(c), (d)].

The outer layer of calloid from all ASW concentrations resembled a uniseriate epidermis. Cells in this layer divided anticlinally with respect to the calloid surface except for those giving rise to stomata, those in meristemoid regions [Fig. 3(f)], and those in isolated regions where oblique and periclinal divisions had occurred [Figs 4(a), (b)]. Some sections revealed relatively large multilayered arrays of smaller cells in flat regions of the calloid surface [Fig. 4(a)]. A small number of cells in these regions had large nuclei and were more densely stained than the surrounding tissue.

Notable variations in plastid size, shape and amount of starch present per plastid were observed within and between cultures [Figs 3(c), (f)]. Many cells of calloids grown in the presence of 100 mOs ASW had either very reduced or degenerated plastids [Fig. 3(e)]. Plastid size at 300 and 350 mOs ASW also appeared to be very small [4(c), (d)] relative to other cultures [Figs 3(c), (f), 4(a)].

## DISCUSSION

Although the development and breeding of improved genotypes of crops is one of the most important potential applications of tissue culture,[18] a number of technical and theoretical problems remain to be solved. Numbers and qual-

FIG. 3. Scanning electron and light microscope images of calloids from standard and artificial seawater-containing cultures. (a) Region from a calloid grown on semi-solid Linsmaier–Skoog medium 300 mOs artificial seawater. Small protocorm-like bodies (p) are beginning to form at the base of the larger organ. × 119. (b) Surface of a calloid grown in liquid Linsmaier–Skoog medium containing 350 mOs artificial seawater. Stomata (s) are visible on the surface of the structure. × 204. (c) Longitudinal section, through a structure similar to the one shown in Fig. 3(b). This tissue was grown on standard semi-solid Linsmaier–Skoog medium. A vascular strand (v) is present in the tissue and numerous rounded amyloplasts (arrows) are visible. × 128. (d) Longitudinal section, through a linear-shaped organ similar to the one shown on Figs 1(b) and 2(c). This tissue was grown on semi-solid Linsmaier–Skoog medium containing 100 mOs artificial seawater. A vascular strand (v) is present with associated dark-stained cells which are tannin-containing idioblasts (d). × 85. (e) Section cut perpendicular to the calloid surface. The calloid was grown on semi-solid Linsmaier–Skoog medium containing 50 mOs artificial seawater. Part of a linear-organ (o) can be seen on the calloid surface. × 128. (f) Section cut perpendicular to the calloid surface. The calloid was grown on semi-solid Linsmaier–Skoog medium containing 100 mOs artificial seawater. Amyloplasts (arrows) are visible in most cells. A uniseriate outer layer (e) appears fairly regular, but some regions (r) have undergone subsequent periclinal and oblique divisions. These areas may represent arrested meristemoid activity as serial sections did not reveal large numbers of meristematic cells in adjacent regions. The dark-stained cells (d) are tannin-containing idioblasts and are often associated with vascular tissue [see Fig. 3(d)]. × 128.
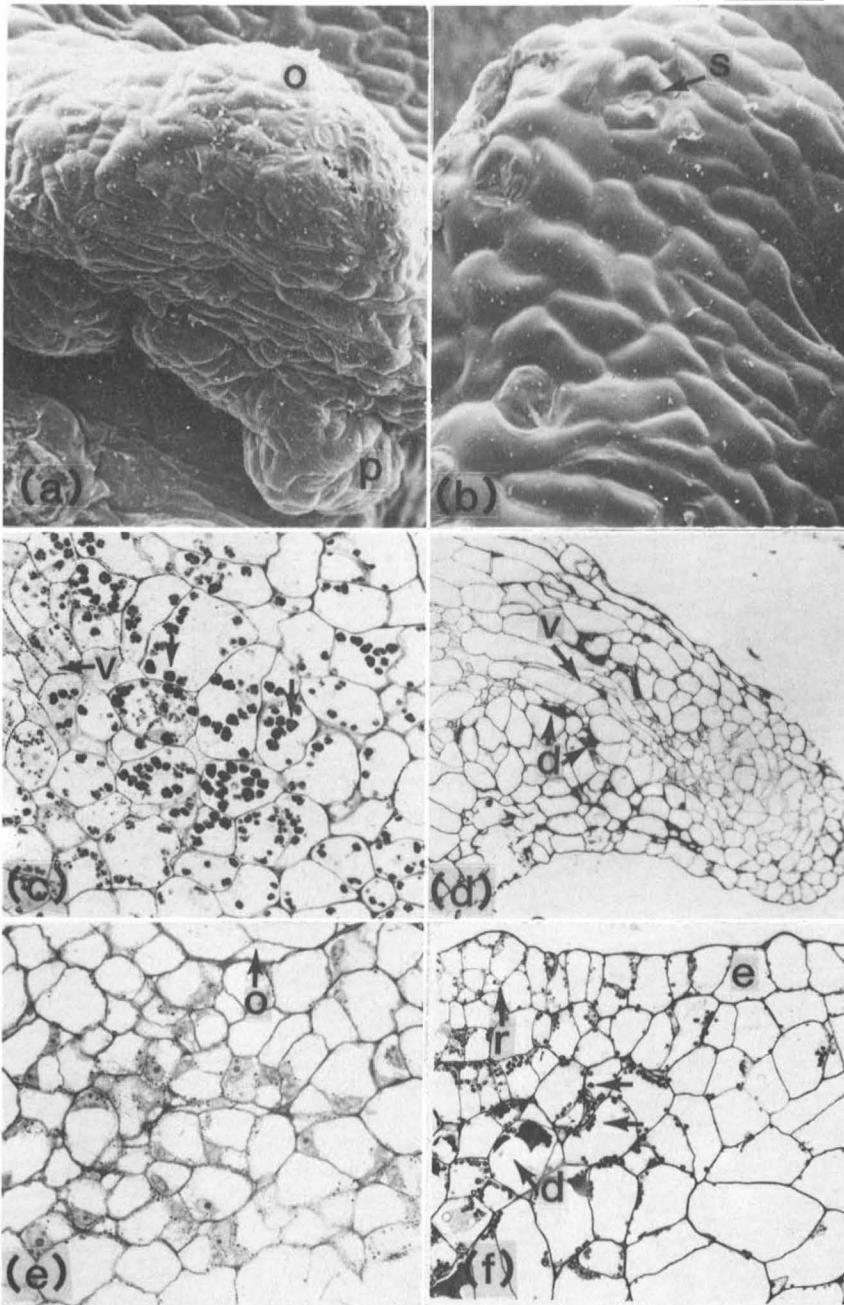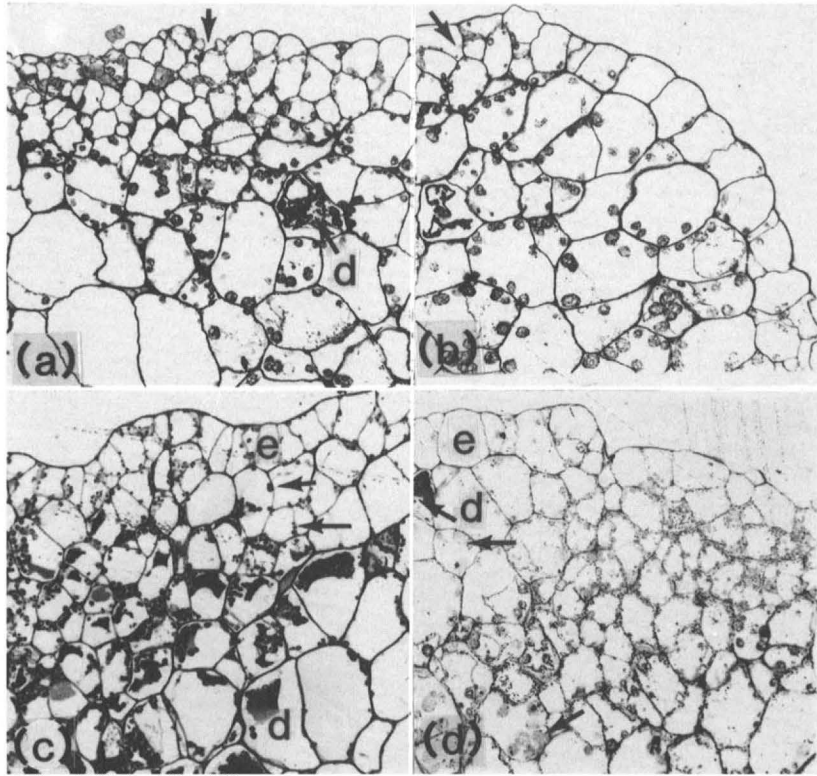
FIG. 3.

FIG. 4. Light microscope images of sections of the calloid surface from tissues grown in artificial seawater-containing media. (a) Section cut perpendicular to the calloid surface from tissue grown on semi-solid Linsmaier–Skoog medium containing 100 mOs artificial seawater. A small pocket of cells (arrows) with oblique and anticlinal divisions disrupts the uniseriate outer layer surrounding it. This could represent a region close to an active meristemoid or an area which had a short period of meristematic activity which has been lost. Tannin idioblasts (d) can be seen in the ground tissue surrounded by vacuolate parenchyma cells containing amyloplasts. × 128. (b) Section cut perpendicular to the calloid surface in a region similar to the one shown in Fig. 3(b). Tissue was grown on semi-solid Linsmaier–Skoog medium containing 150 mOs artificial seawater. A disruption in the uniseriate appearance of the outer calloid layer is present (arrow). × 128. (c) Section cut perpendicular to the calloid surface. This tissue was grown on semi-solid Linsmaier–Skoog medium containing 300 mOs artificial seawater. Many tannin-containing idioblasts are visible (d) and numerous small or degenerating plastids (arrows) are present in the ground parenchyma cells. The outer layer (e) appears to be primarily uniseriate. × 128. (d) Section cut perpendicular to the calloid surface. This tissue was grown on semi-solid Linsmaier–Skoog medium containing 350 mOs artificial seawater. A tannin-containing idioblast (d) is present, a uniseriate outer layer (e), and a number of light stained plastids (arrows) are visible in ground parenchyma cells. × 128.

ity of plantlets produced from callus subcultured for more than a few months may diminish in some species, whereas other plants like taro [7,8] and alfalfa[12] actually require long culture periods for plantlet development. The physiological constraints on rapid plantlet production and the mechanisms which control development (i.e. organogenesis or embryogenesis) are often unknown. Since prolonged culture of tissues may lead to spontaneous genetic changes,[3] the time required for plantlet production becomes an important consideration in determining the strategy and outcome of *in vitro* selection.

Even when *in vitro* selection pressures can be used successfully to produce cells or tissues tolerant to an introduced factor, there are differences in the degree to which traits expressed in culture can be transferred to plantlets generated from them. Success of *in vitro* selection appears to depend both on innate qualities of the tissues used and on the character(s) studied.[4,5,11,14,17,19]

Most studies of salinity effects have been limited to the biochemical, physiological or cytological responses of plant tissues exposed to increasing concentrations of NaCl.[13] Exogenously supplied mixtures of salts are more likely to approximate field conditions than single salts. Hence responses in cultured tissues exposed to increasing concentrations of seawater may be more reliable indicators of potential adaptation to natural saline conditions. Cell and tissue responses are useful in assessing the components of the physiological adaptation to saline conditions, but cannot always be used as a measure of successful plant establishment in soil at comparable levels of salts. Therefore it is important when using *in vitro* systems to understand the mechanism(s) of plantlet regeneration in standard conditions, and the manner in which salinity affects normal plantlet development.

Initial studies of small numbers of taro calloid cultures on standard media[7] did not reveal the regions responsible for plantlet development. Observations of ASW cultures of taro made in this study helped to clarify the nature of *in vitro* plantlet initiation and growth in taro cultures. Time-dependent changes in production of lateral organs on calloid meristemoids (i.e. from linear through spatulate, and finally hastate or peltate forms resembling leaves of seedling-grown

plants), became clear only after large numbers of ASW cultures were examined.

Observations of tissues from all media indicate that plantlets, abnormal leaves and shoots, as well as teratoma-like structures, arise from analogous meristematic regions. Further, the development of meristemoids is dependent upon tissue age, exogenous growth regulator supply and most probably local endogenous supplies of growth active substances. This explanation might account for the heterogeneous appearance of calloid tissues, the diversity of differentiated organs observed, and the increase in normal plantlet production with aging and tissue crowding. The multilayered arrays of cells noted in the calloid surface may represent meristemoids which have become inhibited early in their development.

The differentiation of a recognizable epidermis with well developed stomata appeared to be correlated with and/or a reflection of a more rapid transition to normal plantlet development (e.g. in 150–250 mOs ASW). No other major qualitative differences in general structure were detected in calloid over the range of ASW concentrations used. For example dark stained idioblasts occurred in the ground tissue of all calloids. These were similar to cells observed in taro leaves.[15]

Previous reports[16] and more recent studies in our laboratory[9] indicate that salinity may affect quantitative characteristics and ultrastructural features of cells. Such effects may include variations in cell size, wall thickness, chloroplast size and structure, numbers and size of starch grains and the appearance of the nucleus.

The similarities in morphological and anatomical features of calloids grown on 0 and 50–350 mOs ASW suggest that other than selection for increased tolerance to salinity, growth in ASW is not associated with gross phenotypic transformation of the tissues. This conclusion is supported by studies of growth kinetics[7] and the organic constituents of the tissue.[6] It seems clear therefore that the general problems related to plantlet development are a function of calloid growth rather than ASW concentration. However, both abscisic acid and endogenous cytokinin levels within plants can be affected by exposure to saline conditions.[10] Therefore greater production of tumor-like formations in taro and tissue death at very high ASW concentrations

may be related at least in part to changes in endogenous hormone levels.

Studies to determine the nature of the observed anomalous and slow plantlet growth from calloid cultures are continuing in the hope of clarifying the mechanism(s) involved. *In vitro* selection of ASW tolerance does not appear to significantly alter patterns of organogenesis in taro tissues. Therefore this system holds considerable promise as a means for the production of salt tolerant strains of taro.

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