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"BUDDING" AND NUCLEAR DIVISION IN CULTURED PROTOPLASTS OF CORN, CONVULVULUS, AND ONION¹

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ABSTRACT

Protoplasts were isolated from seedlings of *Zea mays* L., roots of *Convolvulus arvensis* L., and root callus of *C. arvensis* L. (filamentous strain of root callus) and *Allium cepa* L. by cellulase treatment. They were maintained for over 3 weeks in liquid medium containing either 0.4 M sorbitol or a mixture of sorbitol and 1% sucrose. Protoplasts of *C. arvensis* L. were observed to form protuberances or "buds"; such protoplasts demonstrated regenerated wall formation within 2-3 days of culture. A few protoplasts exhibited nuclear divisions on the second day of culture. About 4%-5% became binucleate, and 2% became three- to four-nucleate. In experiments on the effect of "Sendai" virus on plant protoplasts, no agglutination or fusion was observed.

Introduction

Methods are now available which allow the preparation of isolated protoplasts of plant cells from a wide variety of tissues, including seedling organs (COCKING 1960), immature fruits (GREGORY and COCKING 1965), leaves (TAKEBE, OTSUKI, and AOKI 1968; OTSUKI and TAKEBE 1969) and cultivated callus tissues grown in vitro (ERIKSSON and JONASSON 1969; SCHENK and HILDEBRANDT 1969; KAO, KELLER, and MILLER 1970). Studies using isolated protoplasts have been made of the properties of the protoplast membranes (LEVITT, SCARTH, and GIBBS 1936; VREUGDENHIL 1957; COCKING 1961; RUESINK and THIMANN 1965), the uptake of macromolecules and viruses (COCKING 1965, 1966b, 1970), the phenomenon of cell wall regeneration (COCKING 1966a; POJNAR, WILLISON, and COCKING 1967), and protoplast fusion (POWER, CUMMINS, and COCKING 1970; KELLER et al. 1970).

Most reports emphasize the problem of stability of protoplasts in culture, and few accounts describe protoplasts which remained intact and viable for periods greater than a few days. ERIKSSON and JONASSON (1969) reported nuclear division in cultured protoplasts of *Haplopappus gracilis* maintained in a living state for up to 2 weeks. Recently, KAO et al. (1970) were able to produce multicellular colonies from isolated cultured protoplasts derived from cell suspensions of soybean.

In working with the culture of protoplasts it is clear that attention must be paid to two major aspects. First, effective isolation procedures must be achieved, involving cell plasmolysis followed by enzymatic degradation or partial breakdown of the

cell wall leading to protoplast release; second, cultural conditions must be found for maintenance of the protoplasts and provision of the appropriate osmotic conditions, nutrients, and stimuli for cellular proliferation. In this study, special attention was paid to the latter features with a view to achieving more prolonged survival of protoplasts in culture and their multiplication.

Material and methods

Protoplasts were isolated from various sources, including seedling roots and mesocotyls (*Zea*), isolated root tips grown in culture (*Convolvulus*), and callus tissues grown in agar or liquid culture (*Convolvulus* and *Allium*). In table 1 the sources of tissues and the media used for protoplast isolation and culture are listed. With respect to cultured tissues, it was found most effective to maintain the protoplasts in the same nutrient medium used for organ or tissue culture, modified in each case by the addition of the plasmolyticum.

Seeds of *Zea mays* L. were surface sterilized with a 5% solution of Pittchlor (commercial sodium hypochlorite), washed with sterile distilled water, and germinated aseptically in the dark. When the roots were 2-3 cm long, sections were cut from elongating portions of the root and mesocotyl and subjected to enzyme treatment. Root tips of *Convolvulus arvensis* L., which have been maintained for many years in our laboratory (TORREY 1958), were cultured in 125-ml Erlenmeyer flasks containing 50 ml of liquid medium on a rotary shaker (80 rpm) in the dark at 24 C. Root segments immediately proximal to the root tip were used as the source of protoplasts. Root calluses of *C. arvensis* L. and *Allium cepa* L. which had been subcultured regularly on agar nutrient media for several years were employed as sources of protoplasts.

All tissues from these different sources were

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TABLE 1
TISSUES AND MEDIA FOR PROTOPLAST CULTURE

Species	Type of tissue used	Medium used for isolation ^a and culture of protoplasts
<i>Zea mays</i> L. var. Barbecue	Seedling root and mesocotyl segments	Modified LINSMAIER and SKOOG (1965)
<i>Convolvulus arvensis</i> L.	Cultured root tips	Modified Bonner (TORREY 1958)
<i>C. arvensis</i> L.	Root callus (filamentous strain)	M6-3 (TORREY 1968)
<i>Allium cepa</i> L.	Root callus	S2M (TORREY and FOSKET 1970)

^a Isolation medium was usually supplemented with 0.4 M sorbitol and 5% cellulase with sucrose omitted.

handled in essentially the same way. Five percent cellulase "Onozuka 1500" derived from *Trichoderma viride* (supplied by the All Japan Biochemicals Co., Ltd., Nishinomiya, Japan) was dissolved in liquid nutrient media that lacked sucrose but had 0.4 M sorbitol added (see table 1). The solution was filtered through a Millipore filter (0.45 μ pore size) and thereafter handled as a sterile solution. The tissue pieces were placed in a few drops of the solution and incubated at 29 C, overnight, for callus tissue, or 3–4 hr for root and mesocotyl pieces. After incubation the tissue pieces were washed with the same medium, lacking enzyme, to remove all traces of enzyme. A small tissue piece was then placed in a few drops of the medium in a watch glass and teased gently with sterile needles. The protoplasts which were released tended to sink. The supernatant solution was removed with a fine glass pipette, and fresh medium was added. This procedure was repeated a few times until all the debris was removed and a clear suspension of protoplasts was obtained. A few drops of the suspension were poured into a sterile Unitron cavity slide and sealed with a circular cover glass, with sterile mineral oil used around the edges and with care taken to introduce an air bubble during the process. The slides were stored in sterile petri dishes in the dark and examined from time to time with a Unitron inverted phase research microscope.

Besides sorbitol, sucrose, glucose, and mixtures of sorbitol and sucrose were also tested as osmotica. Some experiments were made to test the effect of lowering the osmotic pressure of the final growth medium.

Results and discussion

MAINTENANCE OF PROTOPLASTS.—The process of protoplast isolation involves two steps. The raised osmotic concentration of the medium leads to plasmolysis and the formation of spherical protoplasts within the cell walls—an essential first step in the process. Although such plasmolysis could be achieved with supplements of sorbitol as low as 0.25 M, it was found that the greatest protoplast release could be effected by the addition of 0.4 M

sorbitol to the nutrient medium. The concomitant enzyme treatment weakened the cell wall sufficiently to allow release of the spherical protoplasts. The process of release was assisted by mechanical teasing of the tissue piece, which disrupted the already weakened walls and effected protoplast release.

In general, callus tissues required a longer period of enzyme treatment than did seedling tissues. Although it was more difficult to obtain protoplasts from callus tissues than from seedling tissues, it was found that protoplasts from callus tissues were less vacuolate, showed less tendency to burst, and survived longer in culture. Perhaps this is because callus cells are better adapted to an in vitro environment than cells freshly isolated from the seedling.

In the use of cultured tissues, the age of the callus tissue since the last transfer was important. This point has been considered also by ERIKSSON and JONASSON (1969). Agar-grown callus tissues which had been subcultured in fresh medium about 1 week prior to enzyme treatment produced the best protoplasts. This time coincides with a phase of rapid cell multiplication. Once the tissue had been placed in the enzyme mixture to incubate, the cultures were maintained in the same medium throughout the experiment to reduce chemical shock. After release, the protoplasts were washed in fresh medium to remove the enzyme and then cultured in the medium with the high concentration of sorbitol. Attempts to transfer the protoplasts back to a medium with an osmotic concentration closer to or equal to the original nutrient medium were largely unsuccessful, leading to bursting. Apparently, however, the high osmotic pressure of the medium is no block to mitosis (NAGATA and TAKEBE 1970).

Through use of these procedures, almost all the cells yielded viable protoplasts, which were spherical and uninucleate (fig. 7). With phase optics, the structures of the cells were clearly evident. The protoplasts typically showed cyclosis of the cytoplasm together with a slow rearrangement of organelles and granules and with changed shapes and sizes of the optically less dense vacuoles. In many cases, such

protoplasts were observed to remain viable and healthy for at least 3 weeks.

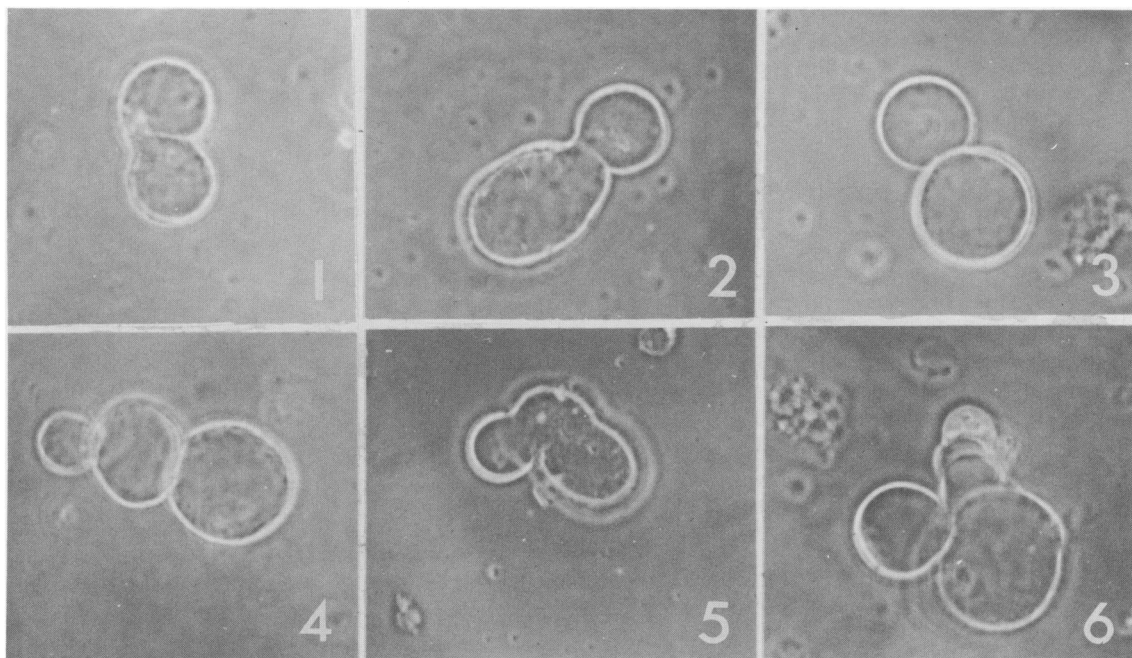
PROTOPLAST BUDDING.—In cultures of protoplasts derived from callus tissues or cultured roots of *Convolvulus*, the phenomenon of protoplast “budding” was encountered. During the first several days in culture, these protoplasts formed new cell walls, as was manifest by a change from the spherical shape accompanied by an increase in size. After about 10–14 days in culture, many of the protoplasts of *Convolvulus* showed localized bulging of the cytoplasm due to a weakening of a part of the cell wall. The cytoplasm protruded because of the internal pressure of the cell and remained distended with a cytoplasmic connection maintained between the mother cell and the protuberance (fig. 1). A new wall formed around the bud; it either remained attached to the mother cell (fig. 2) or became nearly or completely separate (fig. 3). In some cases the process of budding was repeated three or four successive times (figs. 4, 5, 6). Such buds often occurred in a lateral position (figs. 5, 6). In protoplasts with buds, an increase in nuclear number was sometimes observed. Usually the original cell contained a nucleus, and in some cases either the youngest or the intermediate bud also contained a nucleus. Occasionally a newly developed bud would break, and the cyto-

plasm would then retract into the original cell (figs. 11–13).

“Budding” of cells cultured in suspension was found by STEWARD, MAPES, and SMITH (1958) and has been reported also in isolated protoplasts. POJNAR et al. (1967) reported bud formation by tomato root protoplasts, cultured in White’s medium supplemented with coconut milk. Recently HORINE and RUESINK (1970) used budding as an indication of new wall formation in *Convolvulus* protoplasts. They found that bud formation occurred more frequently in darkness than in light and in sucrose medium more frequently than in media containing mannitol or sorbitol. Their ultrastructural study showed that the deposition of new wall-like materials which had been laid down by the protoplast was not like normal wall formation.

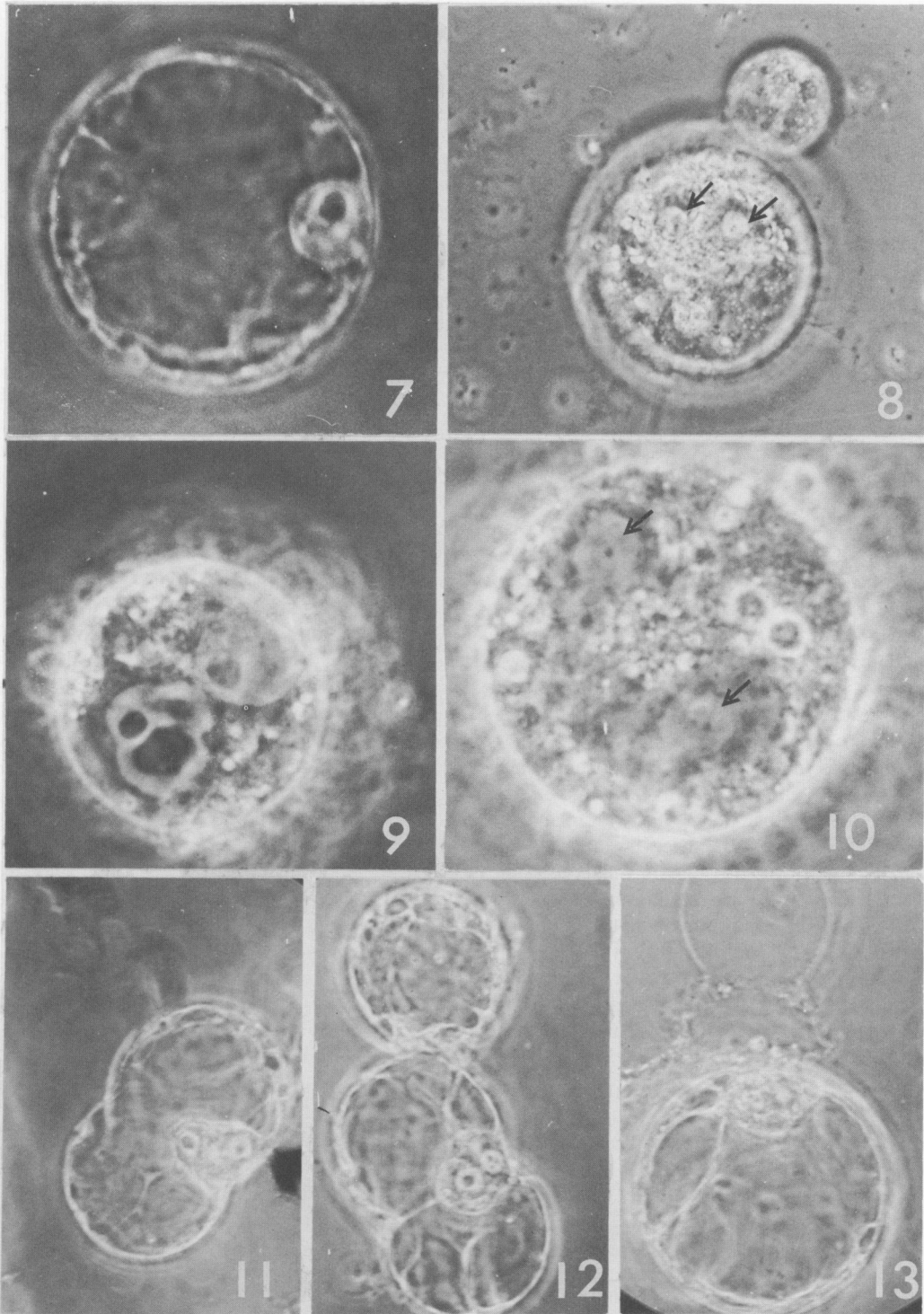
NUCLEAR DIVISION IN PROTOPLASTS.—ERIKSSON and JONASSON (1969) reported an increase in nuclear number in cultured protoplasts of *Haplopappus*. We have made similar observations on cultured protoplasts of *Convolvulus* callus of the filamentous strain and *Allium* callus.

Within 2–3 days of their culture in liquid medium containing 0.4 M sorbitol and the appropriate nutrients, uninucleate protoplasts (fig. 7), which had been isolated from uninucleated cells, showed nuclear



FIGS. 1–6.—Budding phenomenon in root callus protoplasts of *Convolvulus arvensis* L., all $\times 280$. Fig. 1, The protoplast develops a wall around it, a portion of the wall softens, and cytoplasm protrudes. Fig. 2, The protrusion begins to form its own wall. Fig. 3, The bud remains attached to the mother cell

and is enucleate. Fig. 4, Two buds and the mother cell in a row; a nucleus is discernible in the middle cell only. Fig. 5, A lateral bud is formed. Fig. 6, Three successive terminal buds and a lateral bud; nuclei are seen in the mother cell, in the youngest terminal bud and in the lateral bud.



FIGS. 7-13.—Figs. 7 and 9 show the protoplasts of *Allium cepa* L., whereas the rest depict the developmental stages in the protoplasts of *Convolvulus arvensis* L. (filamentous strain of root callus). Fig. 7, Uninucleate protoplast; $\times 1360$. Fig. 8, Binucleate protoplast; two nuclei are lying adjacent to each other (arrows); $\times 560$. Fig. 9, Similar stage; one of the nuclei is lobed and contains three nucleoli; $\times 1360$. Fig. 10, Binucleate protoplast; the two nuclei are apart (arrows) and show aggrega-

tions of granules between them; $\times 1360$. Fig. 11, Four-nucleate protoplast beginning to form a bud; two nuclei are in a different plane of focus; $\times 680$. Fig. 12, Same cell as in fig. 11 at a later stage, showing another terminal bud which is enucleate; $\times 680$. Fig. 13, same cell as in figs. 11 and 12, at a later stage. The mother cell has resumed its original shape, whereas the two buds have burst and their remnants persist; $\times 680$.

division. Mitoses themselves were never observed, but some cytological changes prior to increase in the number of nuclei were observed. Typically, the nucleus itself enlarged, and usually the number of nucleoli increased. By about the third day, 5% of the cultured protoplasts were binucleate with nuclei close to each other (figs. 8, 9). Rarely, nuclei were observed well apart from each other with aggregations of granules between them (fig. 10). No certain cell plate formation was observed in these cultures. Nuclei were observed which were lobed, containing several nucleoli (fig. 9). Spherical cells derived from protoplasts which contained four or six nuclei were also observed. It is believed but not proved that these spherical structures possessed an outer limiting cell wall.

The presence of cell walls around multinucleate protoplasts was clearly evident in cells which underwent budding. Figures 11–13 represent stages in the formation of buds in a *Convolvulus* protoplast which contained four nuclei. In this case, the nuclei remained close together in a dense cytoplasmic mass and showed no tendency to migrate to the new bud which was rich in peripheral cytoplasm. Upon bursting of the buds, the mother cell was able to repair the break and reestablish an intact and viable multinucleate cell.

An attempt was made to improve nutrient conditions to permit normal wall formation between newly formed nuclei. Since no regular wall formation by cell plate development was observed in any of the previously described preparations, it was considered that the total elimination of sucrose from the medium as well as the excessive osmotic concentration of the culture medium might be limiting. We therefore tested a series of media in which 0.4 M sorbitol was replaced by mixtures of 1% sucrose plus levels of sorbitol varying from 0.2 to 0.4 M. In all of these treatments protoplast release occurred but in much

smaller numbers at the 0.2 and 0.25 M sorbitol levels. At 0.3 M sorbitol plus 1% sucrose, optimum protoplast release, survival, and nuclear division were achieved. In every culture established in this medium some protoplasts showed nuclear division by the second day. About 4% became binucleate, and 2% showed three or four nuclei. In week-old cultures an occasional multinucleate cell appeared to have formed a wall, but this fact was not demonstrated conclusively. It appeared, however, from these experiments, that a low level of sucrose, supplementing the plasmolytic levels of sorbitol, was favorable for survival and nuclear multiplication. Normal cell proliferation was not achieved in these conditions. KAO et al. (1970), using a closely similar technique, were able to achieve this type of proliferation from protoplasts derived from soybean cell suspensions.

ATTEMPTS AT PROTOPLAST FUSION.—In animal cell cultures, "Sendai" virus has been used to achieve the fusion of animal cells (HARRIS 1970). We were interested in testing this method on our cultured plant protoplasts. To three to four drops of protoplast suspensions of *Convolvulus*, placed in a Unitron cavity slide, a drop of UV-treated K6 "Sendai" virus concentrate was added. (This virus preparation was kindly provided us by K. HOLMES.) During incubation the slides were agitated intermittently at room temperature and observed at intervals. No agglutination or fusion was seen.

To test the activity of the virus preparation in our culture conditions, a drop of 1% chicken erythrocytes in phosphate-buffered saline (pH 7.2) was added to the protoplast cultures at room temperature. The erythrocytes readily fused together, but there was no fusion of plant protoplasts or of erythrocytes to plant protoplasts for a period of observation of up to 45 min. We concluded that this virus activity does not extend to protoplasts of *Convolvulus* under our conditions of culture.

LITERATURE CITED

- COCKING, E. C. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature* **187**:962–963.
- . 1961. Properties of isolated plant protoplasts. *Ibid.* **191**:780–782.
- . 1965. Ferritin and tobacco mosaic virus uptake; and nuclear cytoplasmic relationships in isolated tomato fruit protoplasts. *Biochem. J.* **95**:28P–29P.
- . 1966a. Electron microscope studies on isolated plant protoplasts. *Z. für Naturforsch.* **21b**:581–584.
- . 1966b. An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. *Planta* **68**:206–214.
- . 1970. Virus uptake, cell wall regeneration, and virus multiplication in isolated plant protoplasts. *Int. Rev. Cytol.* **28**:89–124.
- ERIKSSON, T., and K. JONASSON. 1969. Nuclear division in isolated protoplasts from cells of higher plants grown in vitro. *Planta* **89**:85–89.
- GREGORY, D. W., and E. C. COCKING. 1965. The large-scale isolation of protoplasts from immature tomato fruit. *J. Cell Biol.* **24**:143–146.
- HARRIS, H. 1970. *Cell fusion*. Harvard Univ. Press, Cambridge, Mass.
- HORINE, R. K., and A. W. RUESINK. 1970. Cell wall regeneration around protoplasts from *Convolvulus* tissue culture. *Plant Physiol.* **46** (suppl.):13.
- KAO, K. N., W. A. KELLER, and R. A. MILLER. 1970. Cell division in newly formed cells from protoplasts of soybean. *Exp. Cell Res.* **62**:338–340.
- KELLER, W. A., B. HARVEY, O. L. GAMBORG, R. A. MILLER, and D. E. EVELEIGH. 1970. Plant protoplasts for use in somatic cell fusion. *Nature* **226**:280–282.
- LEVITT, J., G. W. SCARTH, and R. D. GIBBS. 1936. Water permeability of isolated protoplasts in relation to volume change. *Protoplasma* **26**:237–248.
- LINSMAIER, E. M., and F. SKOOG. 1965. Organic growth factor

- requirements of tobacco tissue cultures. *Physiol. Plantarum* **18**:100-127.
- NAGATA, T., and I. TAKEBE. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Planta (Berlin)* **92**:301-308.
- OTSUKI, Y., and I. TAKEBE. 1969. Isolation of intact mesophyll cells and their protoplasts from higher plants. *Plant and Cell Physiol.* **10**:917-921.
- POJNAR, E., J. H. M. WILLISON, and E. C. COCKING. 1967. Cell-wall regeneration by isolated tomato-fruit protoplasts. *Protoplasma* **64**:460-480.
- POWER, J. B., S. E. CUMMINS, and E. C. COCKING. 1970. Fusion of isolated plant protoplasts. *Nature* **225**:1016-1018.
- RUESINK, A. W., and K. V. THIMANN. 1965. Protoplasts from the *Avena* coleoptile. *Nat. Acad. Sci., Proc.* **54**:56-64.
- SCHENK, R. V., and A. C. HILDEBRANDT. 1969. Production of protoplasts from plant cells in liquid culture using purified commercial cellulases. *Crop Sci.* **9**:629-631.
- STEWART, F. C., M. O. MAPES, and J. SMITH. 1958. Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. *Amer. J. Bot.* **45**:693-703.
- TAKEBE, I., Y. OTSUKI, and S. AOKI. 1968. Isolation of tobacco mesophyll cells in intact and active state. *Plant and Cell Physiol.* **9**:115-124.
- TORREY, J. G. 1958. Endogenous bud and root formation by isolated roots of *Convolvulus* grown in vitro. *Plant Physiol.* **33**:258-263.
- . 1968. Hormonal control of cytodifferentiation in agar and cell suspension cultures. Pp. 843-855 in F. WIGHTMAN and G. SETTERFIELD [ed.], *Biochemistry and physiology of plant growth substances*. Runge, Ottawa.
- TORREY, J. G., and D. E. FOSKET. 1970. Cell division in relation to cytodifferentiation in cultured pea root segments. *Amer. J. Bot.* **57**:1070-1080.
- VREUGDENHIL, D. 1957. On the influence of some environmental factors on the osmotic behavior of isolated protoplasts of *Allium cepa*. *Acta Botan. Neerlandica* **6**:472-542.

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THE COMPARATIVE WATER ECONOMY OF REPRESENTATIVE EVERGREEN SCLEROPHYLL AND DROUGHT DECIDUOUS SHRUBS OF CHILE

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ABSTRACT

A comparison was made of the drought responses of four shrubby species occurring in the mediterranean-climatic region of Chile. Two of these species, *Lithraea caustica* and *Kageneckia oblonga*, which are evergreen sclerophylls, showed little tendency to conserve water until the annual summer drought period was considerably advanced. The morphological characteristics and the stomatal behavior of the leaves of these plants indicate features which would limit water loss during periods of drought but not during times of water availability. Two drought deciduous species studied, *Proustia cinerea* and *Flourensia thurifera*, which occur in more xeric habitats than the sclerophylls, responded to the drought primarily by the gradual reduction in leaf surface area of the entire shrub. One of these species, *P. cinerea*, showed no indications of stomatal regulation of water loss right up to the time when leaves abscised during the drought period. In contrast, plants of *Flourensia* had a drought response intermediate between the sclerophyll species and *Proustia*. These plants showed active stomatal control of water loss, but earlier in the drought period than the sclerophylls. As the drought progressed they gradually reduced their leaf surface area. These results are discussed in terms of the carbon balance of these species as it relates to their distribution.

Introduction

In the central region of Chile, between the latitudes of approximately 33°-38° S, the predominant vegetation is an evergreen sclerophyllous scrub (matorral). Average annual precipitation in this region varies between somewhat over 300 to almost 1,500 mm, with a summer drought period ranging from 2 to almost 6 months (MOONEY et al. 1970).

At somewhat lower latitudes, where the annual precipitation is less and the length of the drought period longer, the evergreen scrub gives way to a vegetation which has fewer evergreens and more succulent and drought deciduous elements (MOONEY et al. 1970).

Within the region where these vegetation types overlap, there is the opportunity to study their

comparative response to drought of more or less equal duration. Such a comparative study should lead to an understanding of the adaptive advantages of the various morphological types to drought stress. This information, coupled with a knowledge of the variability and extent of these mediterranean-type climatic regimes, should enable predictions as to where and why certain growth forms prevail along the aridity gradient extending from northern to southern central Chile, as well as comparable gradients in other mediterranean-climatic regions of the world.

The study area

The area selected for study is located near Maipu, Chile, in the Quebrada de La Plata. This small valley is about 30 km southeast of Santiago in the