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Ultrastructure of grape leaf protoplasts in comparison with the source tissue

by

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Ultrastruktur von Rebenblatt-Protoplasten und Ausgangsgewebe — ein Vergleich

Zusammenfassung. — Anhand histochemischer Nachweise, welche das Weiterfunktionieren der Plasmamembran als physiologische Barriere nach dem Isolationsprozeß bestätigen, können Mesophyllprotoplasten von *Vitis vinifera* L. als lebensfähig betrachtet werden. Dieser Befund, sowie das vollständige Fehlen von Zellwandresten, wurde durch elektronenmikroskopische Untersuchungen an Ultradünnschnitten bzw. Gefrierätzproben von Protoplastenpräparaten erhärtet. Das vereinzelte Auftreten von elektronendichten, runden Gebilden (sog. osmiophilic bodies) im Cytoplasma ist vermutlich eine der plasmolysierenden Wirkung des Isolationsmediums zuzuschreibende Erscheinung. Wie früher gezeigt, hat aber die verwendete Isolationsmethodik keinen negativen Einfluß auf den photosynthetischen C-Stoffwechsel der Protoplasten. Daraus geht hervor, daß die lebenswichtigen Zellkomponenten, trotz der isolationsbedingten Streßsituation, keinen irreversiblen und damit nachweisbaren, strukturellen oder funktionellen Beeinträchtigungen unterworfen sind.

Introduction

Along with the increased use of plant protoplasts in investigating somatic hybridization (FOWKE *et al.* 1977, KATJITA *et al.* 1980), cell wall regeneration (WILLISON and GROUT 1978, BURGESS and LINSTEAD 1979), and membrane characteristics (DAVEY and MATHIAS 1979, SCHNABL *et al.* 1980), ample information on the structure and ultrastructural properties of protoplasts has become available in the last few years. Probably because research in this field of interest primarily focuses on the isolated protoplast or protoplast suspension as a functional unit of its own, structural comparisons between intact and disintegrated tissue are scarce (GIGOT *et al.* 1975, TAYLOR and HALL 1978). In addition, the choice of the experimental system has quite legitimately often been influenced by the practicality of the isolation procedure, thus favouring plant material which responded to relatively mild digestive conditions. The introduction of more aggressive cellulase preparations, such as Onozuka R-10, widened the scope of degradable plant tissues. However, even with these more effective enzyme mixtures, in some instances prolongation of the incubation period was necessary. Often also economic considerations concerning laboratory efficiency led to overnight isolation procedures. This inevitably increases the danger of structural damage caused by extended exposure to degrading enzymes (HALL and WOOD 1970), osmotic stress (PRAT 1972), and antibiotics (ROBINSON and SCHLÖSSER 1978), required to avoid bacterial contamination.

The problem of isolating viable protoplasts from leaves of *Vitis vinifera* is further aggravated by the high concentrations in acids and phenolics, which call for appropriate buffering and addition of reducing agents and detergents (POSSNER *et al.* 1981) to the cellulase medium.

Consequently, if protoplast preparations from grape leaves are to be used for physiological experiments (HASLER *et al.* 1982), the principal question of whether the membranes are structurally undamaged, must not be neglected.

The present paper establishes the viability state of isolated grape leaf protoplasts and further provides specific information on their ultrastructure in comparison with that of cells in the intact mesophyll tissue.

Material and methods

Protoplast isolation

Protoplasts were isolated from *Vitis vinifera* (cv. Riesling × Silvaner) by incubating thin leaf slices for 5 h in a solution containing 2 % cellulase Onozuka R-10, 0.35 M mannitol, 0.25 mM morpholinoethanesulfonate, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM KCl, 10 mM cysteamine and 0.4 % polyethyleneglycol. The protoplast suspension was then decanted, filtered and purified on a Ficoll 400 step gradient as described in detail previously (HASLER *et al.* 1982).

Viability tests

The soundness of the plasma membranes was checked by a) mixing equal volumes of the protoplast suspension and a solution of 2.5 % trypan blue in 0.35 M mannitol and b) by adding 20 µl of 5 % fluorescein diacetate to 1 ml purified protoplast suspension. The condition of the vacuole membranes was examined after staining a 70 × *g* protoplast pellet with isotonic 0.1 % aqueous neutral red.

The absence of cell walls or fractions thereof was tentatively established by resuspending freshly isolated protoplasts in isotonic 0.1 % Calcofluor White-M2R solution.

Electron microscopy

a) Sectioning: Isolated protoplast suspensions were centrifuged at 70 × *g* for 10 min. The pellet was subsequently fixed for 1 h at 25 °C with 2.5 % glutaraldehyde in 0.07 M phosphate buffer pH 7.4, rinsed twice without fixative and treated in buffered 4 % osmium tetroxide for another hour. Prior to dehydration in acetone, the protoplast sediment was mixed with 3 % agar solution heated to 60 °C and allowed to solidify. After dehydration, the agar blocks were embedded according to SPURR (1969).

Small pieces of grape leaves were handled in the same way, with fixation in glutaraldehyde and osmium tetroxide for 2 h each, but omitting the agar stabilization.

The embedded samples were cut with a diamond knife in a LKB Ultrotome III and the sections stained in 2 % aqueous uranylacetate.

b) Freeze-etching: Protoplasts were frozen onto 3 mm collared gold specimen mounts by immersion in nitrogen slush. Freeze-etching was done in a Balzers BA 360 device. The samples were etched for 2 min at −100 °C. PT/C shadowing material was evaporated from an electron beam source and the thickness of coating controlled by a quartz crystal monitor. The replicas were cleansed overnight in 70 % sulphuric acid and washed extensively in distilled water.

The specimens were finally mounted on Formvar-coated grids and examined in a Hitachi HS-8 electron microscope at 50 kV.

Results

Because the phenomenon of protoplast cyclosis and the soundness of the plasmalemma are known to be intrinsically related, only preparations showing a negligible number of nonspherical individuals after filtration (Fig. 1 a), were submitted to further

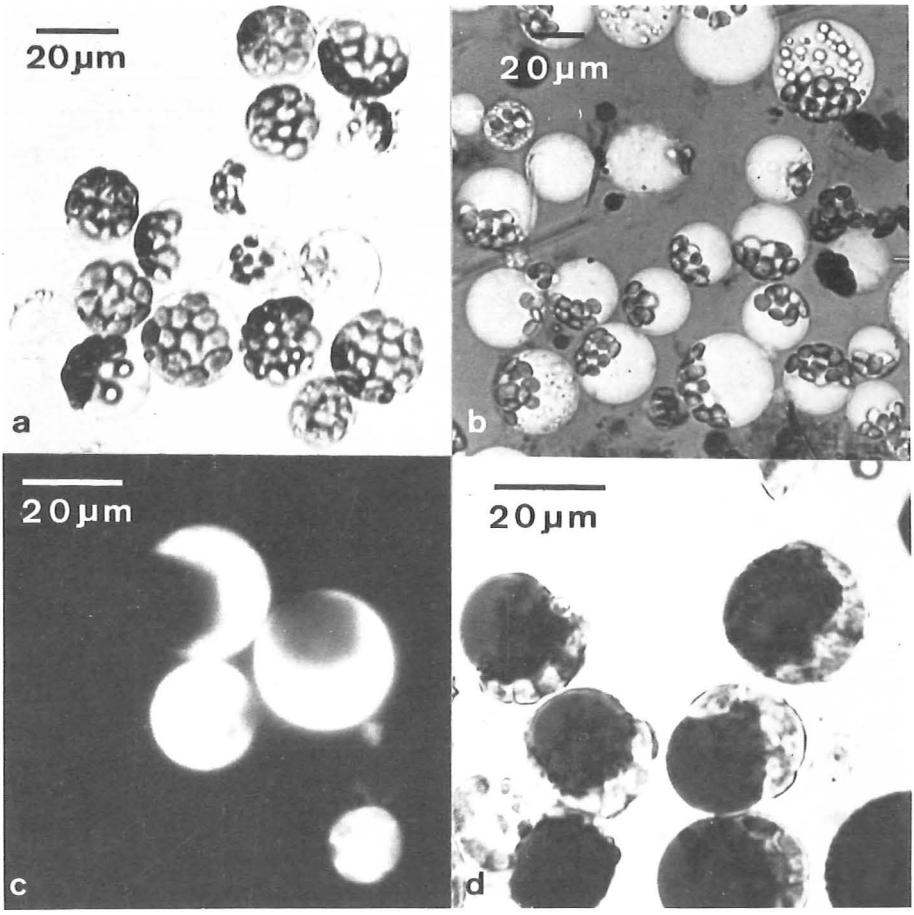
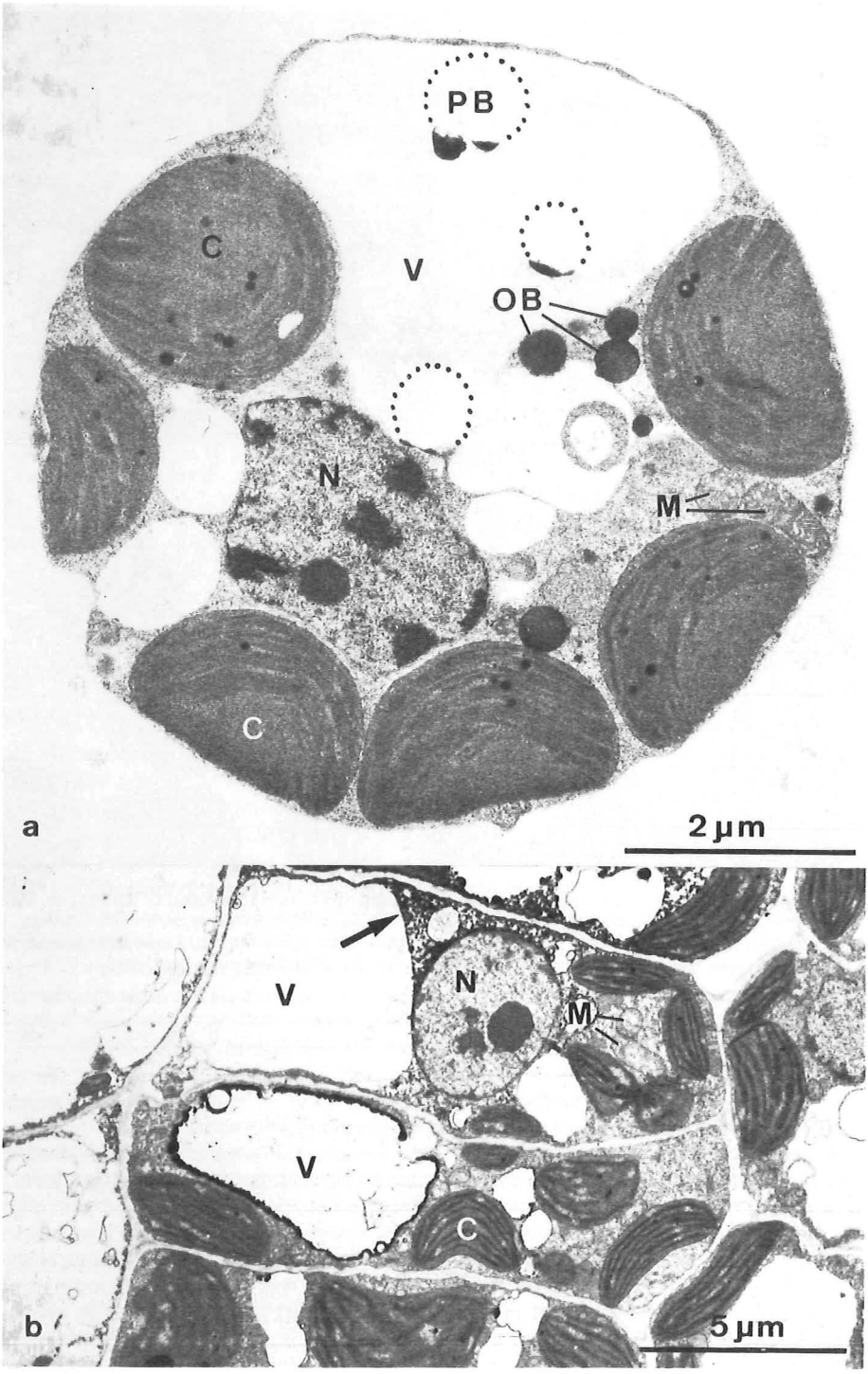


Fig. 1: Light micrographs of grape leaf protoplasts submitted to variable viability tests: a) suspension of mesophyll protoplasts showing the crucial phenomenon of cyclosis; b) protoplast suspension after staining with trypan blue; c) fluoresceing cytoplasm after application of fluorescein diacetate; d) protoplasts accumulating neutral red within their vacuoles.

Mikroskopische Aufnahmen von Rebenblatt-Protoplasten: a) Protoplasten mit eingetretener Zyklose; b) Suspension nach Färbung mit Trypanblau; c) Cytoplasmafluoreszenz nach Zugabe von Fluoresceindiacetat; d) Protoplasten nach Aufnahme von Neutralrot in die Vakuolen.

treatment. The protoplast diameter was found to range between 8 and 32 µm, with a maximum frequency at approximately 20 µm. The normal functioning of the plasmalemma was established by checking its potential to differentiate between trypan blue, a vital stain which is excluded by intact membranes (Fig. 1 b; KANAI and EDWARDS 1973) and fluorescein diacetate, which is not. The latter dye can subsequently be localized by the UV fluorescence of its degradation products (Fig. 1 c), formed upon enzymic cleavage in the cytoplasm (LARKIN 1976). Tonoplast condition was checked by resuspending sedimented protoplasts in 0.35 M mannitol solution at pH 7, containing 0.1 % neutral red. The dye permeates the cellular membranes, but upon entering the acidic environment of the vacuole it is positively charged and accumulates inside (STADELMANN and


PB
C
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KINZEL 1972). Because protonation is accompanied by the appearance of a bluish-red colour, the substance can be easily located by light microscopy (Fig. 1 d).

No fragments of cell walls or cellulose strands could be detected on isolated protoplasts by staining with Calcofluor White-M2R (NAGATA and TAKEBE 1970), nor did electron micrographs of protoplasts show any wall remnants (Fig. 2 a) of fibrous material attached to the plasmalemma (Fig. 3).

The most prominent change in leaf cell ultrastructure after isolation as protoplasts (Fig. 2 a) is the occurrence of amorphous, osmiophilic bodies in the cytoplasm (GIGOT *et al.* 1975, TAYLOR and HALL 1978). Similar, but smaller, dark droplets are observed in grape leaf chloroplasts before and after disintegration of the tissue (Fig. 2 a and b).

Because actinomycin D, which was used in our isolation procedure to prevent microbial growth, has been reported to cause structural aberrations, such as inflations of the nuclear membrane, folding of the endoplasmatic reticulum and changes in mitochondrial and chloroplast shape (ROBINSON and SCHLÖSSER 1978), special attention was directed towards this type of alterations. However, all membrane structures were found to remain normal throughout protoplast isolation (Fig. 2 a).

There may exist a relationship between the electron-dense layers, occasionally found coating the tonoplast (Fig. 2 b), which are believed to consist of phenolic compounds and the dark, spheroid bodies in protoplast vacuoles. However, comparable structures could not be detected in freeze-etched protoplasts revealing the inside of the vacuole.

The only remarkable anomaly detected was an incidental occurrence of protoplast fusions. Multinucleate individuals with up to a maximum of seven nuclei in one section were found. It seems unlikely that serial nuclear divisions are responsible for this phenomenon, because also the number of chloroplasts and mitochondria was increased. Therefore, the presence of fusiogenic agents such as polyethyleneglycol 4000, Na^+, K^+, Mg^{++} and Ca^{++} (WALLIN *et al.* 1974), although in comparatively low concentrations, was originally thought to account for these prodigies. However, on close examination it was found that fused individuals were rarely observed in crude preparations and must consequently be attributed to the use of Ficoll 400 in the purification step, possibly in combination with the forced physical contact between the protoplasts during centrifugation.

Fig. 2: Comparison between the ultrastructure of isolated protoplasts and intact mesophyll from *Vitis vinifera:* a) electron micrograph of a typical protoplast. Structure and number of organelles correspond to the situation in the source tissue. The localization of the dark, spheroid bodies of presumably phenolic nature, which were found in some protoplast vacuoles, is indicated by dotted lines. Due to structural inhomogeneity, part of these globules was torn out during sectioning. Somewhat smaller osmiophilic bodies appear in the cytoplasm; b) intact grape mesophyll tissue. The phenolic substances either line the margin of the vacuole or, after having leaked out of their compartment during fixation, caused denaturation and darkening of the cytoplasm (arrow). — Abbreviations: C chloroplast, N nucleus, V vacuoles, M mitochondria, PB "phenolic" bodies, OB osmiophilic bodies.

Vergleich der Ultrastruktur eines isolierten Protoplasten mit derjenigen von intaktem Mesophyllgewebe des Weinrebenblattes: a) Elektronenmikroskopische Aufnahme eines typischen Protoplasten. Struktur und Organellzahl entsprechen den Verhältnissen im Ausgangsmaterial. Die Umrisse der dunklen, vermutlich phenolischen Kugeln in der Vakuole sind mit Punkten deutlich gemacht, da diese wegen des Konsistenzunterschieds beim Schneiden herausgerissen wurden. Kleinere osmiophile Körper treten auch im Cytoplasma auf; b) Intaktes Rebenmesophyll. Die phenolischen Substanzen sind entweder entlang der Vakuolengrenze abgelagert oder bewirken — sofern sie während der Aufarbeitung austreten — eine Denaturierung des Cytoplasmas (Pfeil). — C Chloroplast, N Kern, V Vakuolen, M Mitochondrien, PB „phenolische" Körperchen, OB osmiophile Körperchen.

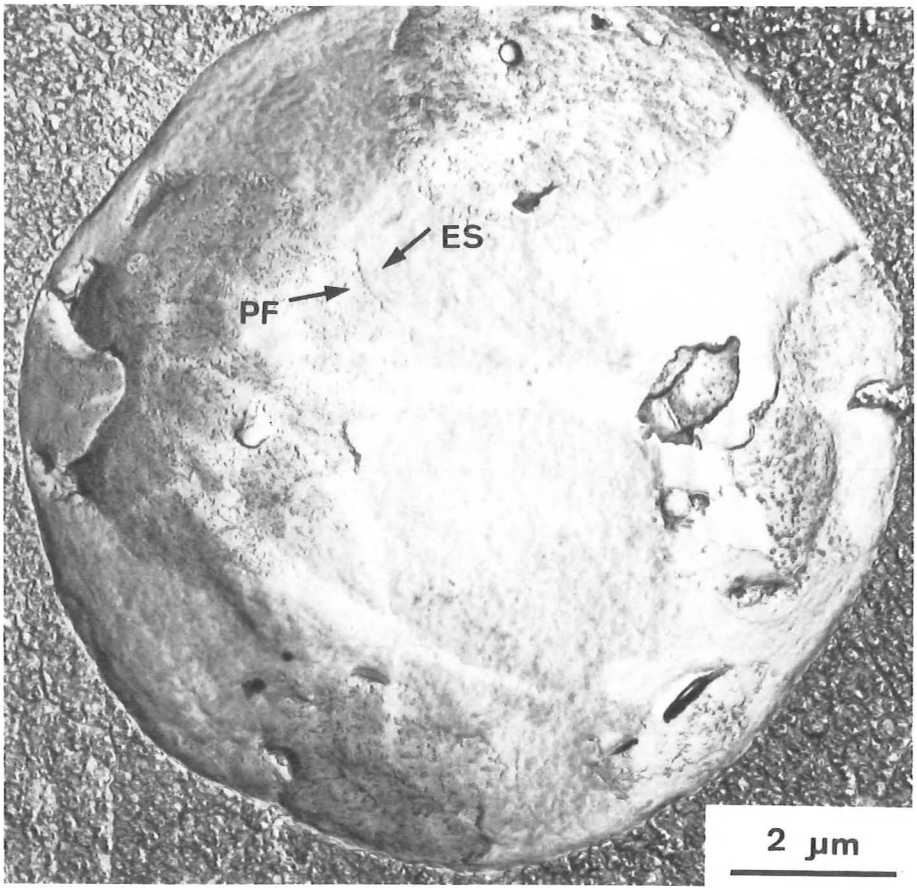

Fig. 3: Electron micrograph showing a freeze-etched grape mesophyll protoplast. The arrows, marked ES and PF, indicate the exposition of the respective fracture face.

Elektronenoptische Aufnahme eines mit Gefrierätztechnik präparierten Protoplasten aus Rebenmesophyll. Die mit ES und PF angeschriebenen Pfeile markieren Ansichten der betreffenden Bruchflächen.

Discussion

The physiological state of the source plant material is known to exert considerable influence on the susceptibility of cell walls to cellulase treatment (HALL and WOOD 1970). Accordingly, preparations from grape leaves grown e.g. in direct sunlight or from mildew-infested vines, contained high proportions of isolated single cells with apparently intact walls as well as protoplasts, which had not undergone cyclosis, thus indicating that the membranes had been damaged. Samples from suitable material did not show these aberrations, but consisted essentially of perfectly round and normal sized individuals, which responded to vital staining tests as expected (Fig. 1 a-d). Once the cytochemical properties were established, the possibility of cell wall fragments or undigested fibres still clinging to the outer surface was checked and found to be nega-

tive. However, because the sensitivity of the indicator used in this study has been questioned in the past (WILLIAMSON *et al.* 1977), the ultrastructure of the plasmalemma was investigated. Inspections of ultrathin sections of protoplasts (Fig. 2 a) confirmed the above result, and also freeze-etching replicas were found to be devoid of fibrous material (Fig. 3). Depending on the fracture plane, the plasmalemma presents itself as a relatively smooth area (ES fracture face; BRANTON *et al.* 1975) or speckled with randomly dispersed particles (PF fracture face).

The most prominent feature of isolated protoplasts in comparison with the source tissue, namely the occurrence of so-called osmiophilic bodies, seems particularly ambiguous. As the phenomenon is visualized by a relatively unspecific staining reaction, it appears to be brought about by variable effects and the amorphous, black globules are classified in three groups: 1. Small, electron-dense droplets appearing within chloroplasts (Fig. 2 a) are a normal attribute of these organelles (GREENWOOD *et al.* 1963, BAILEY and WHYBORN 1963) and also occur in intact grape leaves (Fig. 2 b; MILOSAVLIEVIĆ and NICOLIĆ 1974). 2. Vacuolar inclusions of presumably phenolic nature appear either as dark layers, coating the periphery of vacuoles (Fig. 2 b), or as globular particles within this compartment (Fig. 2 a; MÜLLER and GREENWOOD 1978). 3. Occurrence of osmiophilic bodies in the cytoplasm is thought to result from physiological stress, notably plasmolysis, high temperature and pH-effects during protoplast isolation, because the symptom could be induced by submitting leaves to comparable conditions (TAYLOR and HALL 1978).

The greenhouse-grown leaf material used in this study contained no cytoplasmic globuli, while they did occur in some protoplasts. However, the general metabolic pattern of grape leaf protoplasts appears not to be affected by the isolation procedure, as has been demonstrated recently (HASLER *et al.* 1982).

We thus feel confident that our method yields protoplast preparations which collectively represent the original tissue and therefore may be used without hesitation for the isolation of physiologically active protoplasts, cell components or general investigations where sporadic fusions of individual protoplasts are not a major issue.

Summary

Based on their histochemical properties, protoplasts from grape leaf mesophyll were found to be viable and devoid of remnants of the cell wall. The results were confirmed by electron microscopy of sectioned and freeze-etched protoplast preparations. The only remarkable difference between cells in the intact tissue and the protoplasts was the occurrence of electron-dense globules in the cytoplasm of some individuals. These "osmiophilic bodies" are believed to be due to inevitable plasmolytic effects during wall removal and seem not to have any influence on protoplast CO_2-assimilation, as has been demonstrated earlier. It therefore appears that the vital structures remain intact and functional, in spite of the stress situation created by the isolation procedure.

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Literature cited

BAILEY, J. L. and WHYBORN, A. G., 1963: The osmiophilic globules of chloroplasts. II. Globules of the spinach-beet chloroplast. Biochim. Biophys. Acta **78**, 163—174.

BRANTON, D., BULLIVANT, S., GILULA, N. B., KARNOVSKY, M. J., MOOR, H., MÜHLETHALER, K., NORTHCOTE, D. H., PACKER, L., SATIR, B., SATIR, P., SPETH, V., STÄHELIN, L. A., STEERE, R. L. and WEINSTEIN, R. S., 1975: Freeze-etching nomenclature. Science **190**, 54—56.

BURGESS, J. and LINSTEAD, P. J., 1979: Structure and association of wall fibrils produced by regenerating tobacco protoplasts. Planta **146**, 203—210.

DAVEY, M. R. and MATHIAS, R. J., 1979: Close-packing of plasma membrane particles during wall regeneration by isolated higher plant protoplasts — Fact or artifact? Protoplasma **100**, 85—99.

FOWKE, L. C., CONSTABLE, F. and GAMBORG, O. L., 1977: Fine structure of fusion products from soybean cell culture and pea leaf protoplasts. Planta **135**, 257—266.

GIGOT, C., KOPP, M., SCHMITT, C. and MILNE, R. G., 1975: Subcellular changes during isolation and culture of tobacco mesophyll protoplasts. Protoplasma **84**, 31—41.

GREENWOOD, A. D., LEECH, R. M. and WILLIAMS, J. P., 1963: The osmiophilic globules of chloroplasts. I. Osmiophilic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba* L. Biochim. Biophys. Acta **78**, 148—162.

HALL, J. A. and WOOD, R. K. S., 1970: Plant cells killed by soft rot parasites. Nature **227**, 1266—1267.

HASLER, M., RUFFNER, H. P. and RAST, D. M., 1982: High-yield isolation of grape leaf protoplasts as an instrument in physiological research. Experientia **38**, 564—565.

KAJITA, S., MATSUI, C., SYONO, K., SUZUKI, M. and NAGATA, T., 1980: Fine structure of fusion bodies formed between pea root nodule and tobacco mesophyll protoplasts. Z. Pflanzenphysiol. **97**, 233—240.

KANAI, R. and EDWARDS, G. E., 1973: Purification of enzymatically isolated mesophyll protoplasts from C_3, C_4, and crassulacean acid metabolism plants using an aqueous dextran-polyethylene glycol two-phase system. Plant Physiol. **52**, 484—490.

LARKIN, P. J., 1976: Purification and viability determinations of plant protoplasts. Planta **128**, 213—216.

MILOSAVLIEVIĆ, M. and NICOLIĆ, D., 1974: Photosynthese, Aminosäurengehalt und Feinstruktur des Blattparenchyms bei der Weinrebe. Vitis **12**, 306—315.

MÜLLER, W. C. and GREENWOOD, A. D., 1978: The ultrastructure of phenolic-storing cells fixed with caffeine. J. Exp. Bot. **29**, 757—764.

NAGATA, T. and TAKEBE, I., 1970: Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta **92**, 301—308.

POSSNER, D., RUFFNER, H. P. and RAST, D. M., 1981: Isolation and biochemical characterization of grape malic enzyme. Planta **151**, 549—554.

PRAT, R., 1972: Contribution à l'étude des protoplastes végétaux. I. Effet du traitement d'isolement sur la structure cellulaire. J. Microscopie **14**, 85—114.

ROBINSON, D. G. and SCHLÖSSER, U. G., 1978: Cell wall regeneration by protoplasts of *Chlamydomonas*. Planta **141**, 83—92.

SCHNABL, H., VIENKEN, J. and ZIMMERMANN, U., 1980: Regular arrays of intramembraneous particles in the plasmalemma of guard cell and mesophyll cell protoplasts of *Vicia faba*. Planta **148**, 231—237.

SPURR, A. R., 1969: A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**, 31—43.

STADELMANN, E. J. and KINZEL, H., 1972: Vital staining of plant cells. In: PRESCOTT, D. M. (Ed.): Methods in Cell Physiology. Vol. **V**, 325—372. Academic Press, New York, London.

TAYLOR, A. R. D. and HALL, J. L., 1978: Fine structure and cytochemical properties of tobacco leaf protoplasts and comparison with the source tissue. Protoplasma **96**, 113—126.

VALLIN, A., GLIMELIUS, K. and ERIKSON, T., 1974: The induction of aggregation and fusion of *Daucus carota* protoplasts by polyethylene glycol. Z. Pflanzenphysiol. **74**, 64—80.

WILLIAMSON, F. A., FOWKE, L. C., WEBER, G., CONSTABEL, F. and GAMBORG, O., 1977: Microfibril deposition on cultured protoplasts of *Vicia hajastana*. Protoplasma **91**, 213—219.

WILLISON, J. H. M. and GROUT, B. W. W., 1978: Further observations on cell-wall formation around isolated protoplasts of tobacco and tomato. Planta **140**, 53—58.

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