

酵素処理によるイネの葉およびカルスからの原形質体の分離

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Enzymatic Isolation of Protoplasts from the Rice Leaves and Callus Cultures*

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Since it has been indicated the possibility of protoplast isolation from mesophyll and other tissues of higher plants using enzyme solution, the plant protoplasts are becoming to an available material for studying cell wall regeneration, macromolecular uptake, cell fusion and somatic hybrid^{20,23}. Recently, it has been reported that protoplasts can be isolated from various plants, i.e. wheat⁵, corn¹⁶, rye⁵, *Brassica*¹⁴, *Convolvulus*¹¹, *Crepis*²⁹, *Petunia*^{9,27}, and liverwort²⁸, and from various tissues, i.e. monocot leaves^{5,16}, callus tissues^{10,13}, suspended cells²², pollen tetrads¹ and microsporocyte¹². Up till now, however, we have not been aware of any study on the protoplast isolation from tissues of rice plants except that from roots¹⁹.

Therefore, we investigated the method of isolation from rice leaves and callus cultures. In this paper, the suitable condition for effective isolation of rice protoplasts is described. And also size and properties of them are added, being examined their structure in semithin sections lightmicroscopically.

MATERIALS AND METHODS

Rice seeds (*Oryza sativa* L. variety Aichi asahi) were soaked in water at 30°C for 2 days and then seeded on polyethylene sieves. The seedlings were cultured without nutrients at 30°C under continuous fluorescent light. As experimental materials, leaves and roots were excised from the seedlings, aged variously after the seeding, at 4 to 5 cm length. Then, the leaf and root segments were cut into blocks of 0.5 mm³ or less using razor blade and incubated in the media containing several combination of enzymes.

Callus tissues were induced from rice seeds (variety Aichi asahi) by the method described in the previous paper¹⁷. After subculturing for several passages on the agar medium containing Murashige and Skoog's mineral nutrients, casein hydrolysate and 10⁻⁵ M of 2,4-dichlorophenoxyacetic acid, etc¹⁸, materials were excised from various regions of the callus tissues and were cut into 0.5 mm³ or less as in leaves and roots.

Samples of 2 to 3 g in fresh weight were taken out from these excised materials. They were incubated in 20 ml of enzyme solution contained in 100 ml Erlenmeyer flasks. The incubation was carried out in a reciprocal shaker (100 rpm) at 35°C. Composition of enzyme solution tested and treatment time are indicated in table 1. In most of the experiments, we employed mixed enzyme solution, but not successive treatment. In some experiment not shown in the table, cellulase Onozuka P-1,500 was replaced by R-10.

The protoplasts prepared after treatment were collected by filtering through four fold of nylon sieves and centrifuging for 2 min at about 700 g (2,000 rpm) on clinical centrifuge. The collected protoplasts were observed by light microscope and their diameter was sized.

To observe the structure of protoplasts, the semithin sections were prepared. Glutaraldehyde was added to the materials liberated in the enzyme solution of 2 hours treatment, to give a final concentration of 5%. After 3 hours' fixation at 5°C, the materials were washed 3 times with a 0.2 M phosphate buffer at pH 7.5. The protoplasts were collected by centrifuging and embedded in blocks of a 1% final concentration of agar. The agar

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Table 1 Effects of enzyme solution*, incubation time and age of material used on the isolation of rice protoplasts

Conditions used	Leaves		Roots		Calluses	
	tested	suitable	tested	suitable	tested	suitable
Macerozyme (%)	0.5~2.5	0.5	0.5~2.5	1.5	0.5~4.0	1.0
Cellulase (%)**	1.0~7.0	5.0	3.0~10.0	5.0	1.0~9.0	5.0
Dextran sulfate (%)***	0.5~2.0	0.5	0.5~2.0	1.0	0.5~1.0	0.5
Mannitol (M)	0.4~1.0	0.7	0.2~0.8	0.6	0.1~0.7	0.5
Incubation time (hr)	0.5~6.0	2.0	1.0~6.0	2.5	0.5~3.0	2.0
Age after seeding or subculturing (day)	5~17	7	3~14	5	3~33	15~20

* pH 5.5, ** Onozuka P 1500, *** Meito Sangyo Co. LTD.

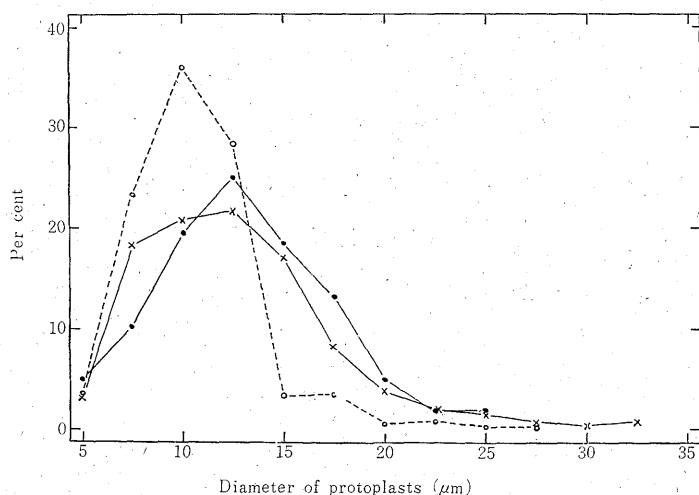


Fig. 1 Size of the protoplasts derived from leaves, roots and callus cultures of rice
 ---○---: leaf protoplast, —×—: root protoplast, —●—: callus protoplast.

blocks were postfixed in a 2% osmium tetroxide in a phosphate buffer at 5°C, overnight, then dehydrated through a graded series of ethanol. To infiltrate epoxy resin, we used propylene oxide following ethanol. The blocks were placed in gelatin capsules. The sections of 0.25 and 0.5 μm in thickness cut on a Porter-Blum MT 1 Ultramicrotome were collected on slide glasses, stained by toluidine blue, fuchsin-methylene blue²¹⁾ (figs. 9, 13 to 18 and 20) or PAS-toluidine blue⁶⁾ (figs. 5 to 8, 10 to 12 and 19) and examined in light microscopy.

RESULTS AND DISCUSSION

The suitable condition for protoplast isolation was researched. The results obtained from the experiments using various treatments are summarized in table 1, in which the tested and suitable concentra-

tions of enzymes and other substances are represented. It is revealed that cellulase has optimum around 5% for degradation of cell walls in all the materials used. While macerozyme is required higher concentration for isolation of root protoplasts than leaf ones. It seems likely that macerozyme is relatively effective for isolation of root protoplasts. Many protoplasts were obtained with even only cellulase treatment from leaves and callus cultures. Higher levels of macerozyme were probably harmful to all the material employed. Mannitol was sufficient for callus protoplasts at lower level such as 0.5 M, but a higher level was required for leaves. Appreciable effects were not obtained in dextran sulfate, but 0.5 to 1.0% of its concentration could be used without deterioration.

In addition, it is certain that the regions, from

which the used materials was taken out, and the age of seedlings and callus cultures are important to isolate the protoplasts readily and abundantly. It seems likely that more protoplasts are occurred from leaf sheaths and young laminae enveloped with them than mature laminae. Many protoplasts were usually produced from the callus cultures between 15 to 20 days. They may be released more certainly from newly formed regions with pale yellow colour than adult regions with black colour. In this respect, it is of interest that the ability of protoplast isolation is different between callus strains obtained from different zones of the carrot root¹³. In our experimental conditions, the protoplasts were scarcely produced at the aged seedlings over 17 days after seeding and the aged cultures over 76 days after subculturing.

Optimal time of incubation was about 2 hours. The prolonged incubation caused large amount of debris and brought about bacterial contamination, because aseptic environment was not complemented. Cutting the materials into small blocks was especially essential for success of the isolation, because the protoplasts were able to be free from the cut surface. Also the enzyme treatment cut away the root hairs occasionally, and the protoplasts seem to be liberated from the cut surface.

In figs. 2 and 4, the protoplasts released enzymatically from rice leaves and callus cultures are indicated, being concentrated by centrifusing.

In fig. 1, frequency distribution of protoplast size is indicated. The diameter of them were estimated at 389, 235 and 364 of protoplasts in leaves, roots and calluses, respectively, and per cent was computed against protoplast size. It is seen clearly from the figure that callus and root protoplasts are larger in their size than leaf protoplasts. Such difference in size may be considered due to osmotic pressures of

enzyme solution, which are optimum in 0.7, 0.6 and 0.5 M of mannitol for isolation of leaf, root and callus protoplasts, respectively.

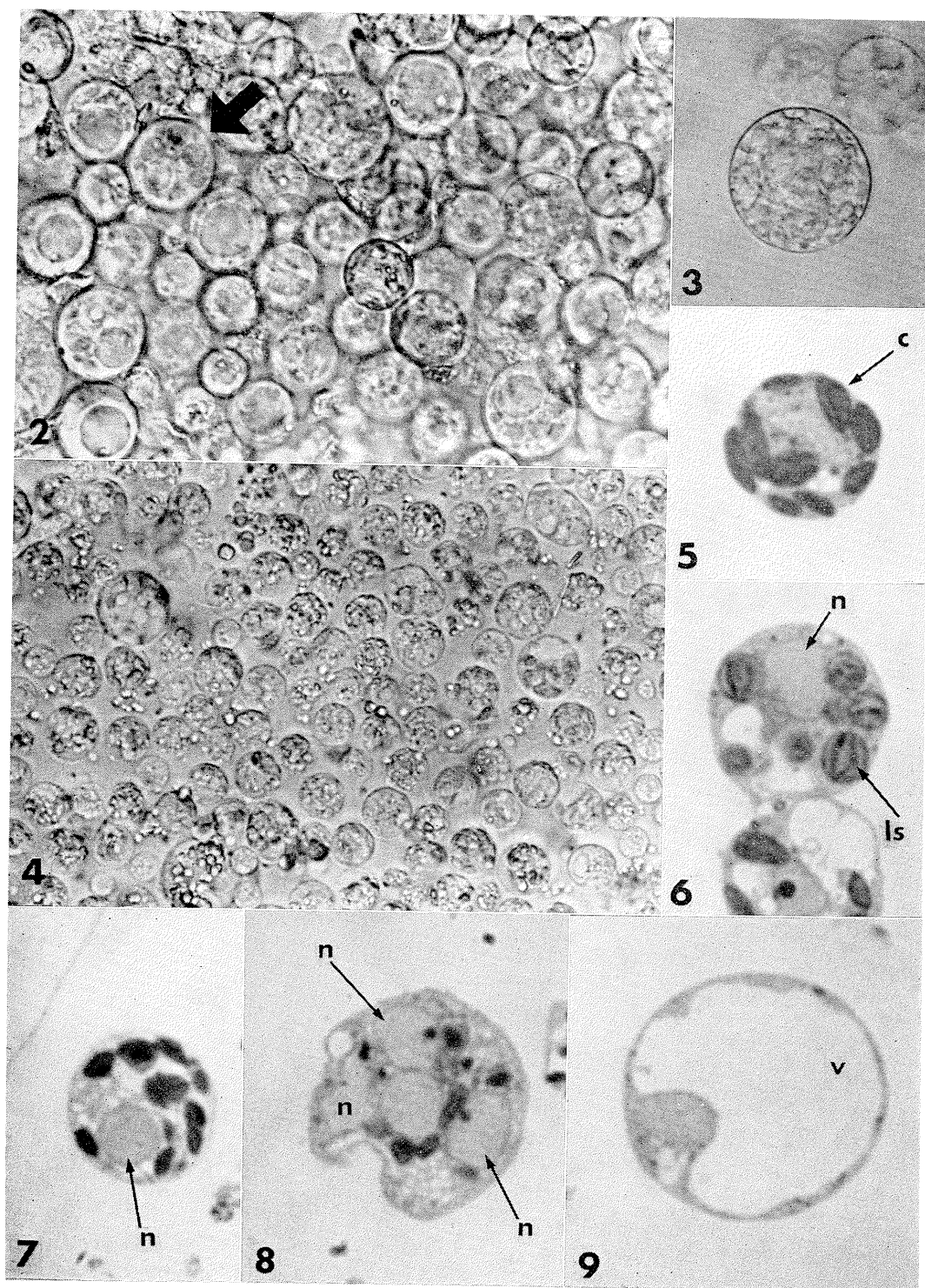
It is ascertained that leaf protoplasts are about 10.3 μm in mean diameter and have relatively homogenous size, yielding their size between 7.5 to 12.5 μm in 87% or more, but observed sometimes a few large protoplasts about 25 μm diameter. Such high frequency seems to be caused by the homogeneity of tissues, from which the protoplasts are isolated. Epidermal, vascular and sclerenchymatous cells were more resistant than mesophyll cells to the enzyme treatment (figs. 10 and 11). Most of the protoplasts liberated from leaves seem to be mesophyll protoplasts, because of having many or few of green chloroplasts (figs. 5 to 7). In fig. 6, lamellar structure can be seen clearly in chloroplasts. The protoplasts without visible chloroplasts (fig. 9) were only 12.8%. Mesophyll cells, of which cell wall was not yet digested by the enzymes, are shown in fig. 12. In this figure, plasmalemma is separated from cell wall by hypertonic solution. It is considered that middle lamella of cell wall in spherical protoplasts is degraded enough, because the envelope of protoplasts was not stained by PAS reaction⁶.

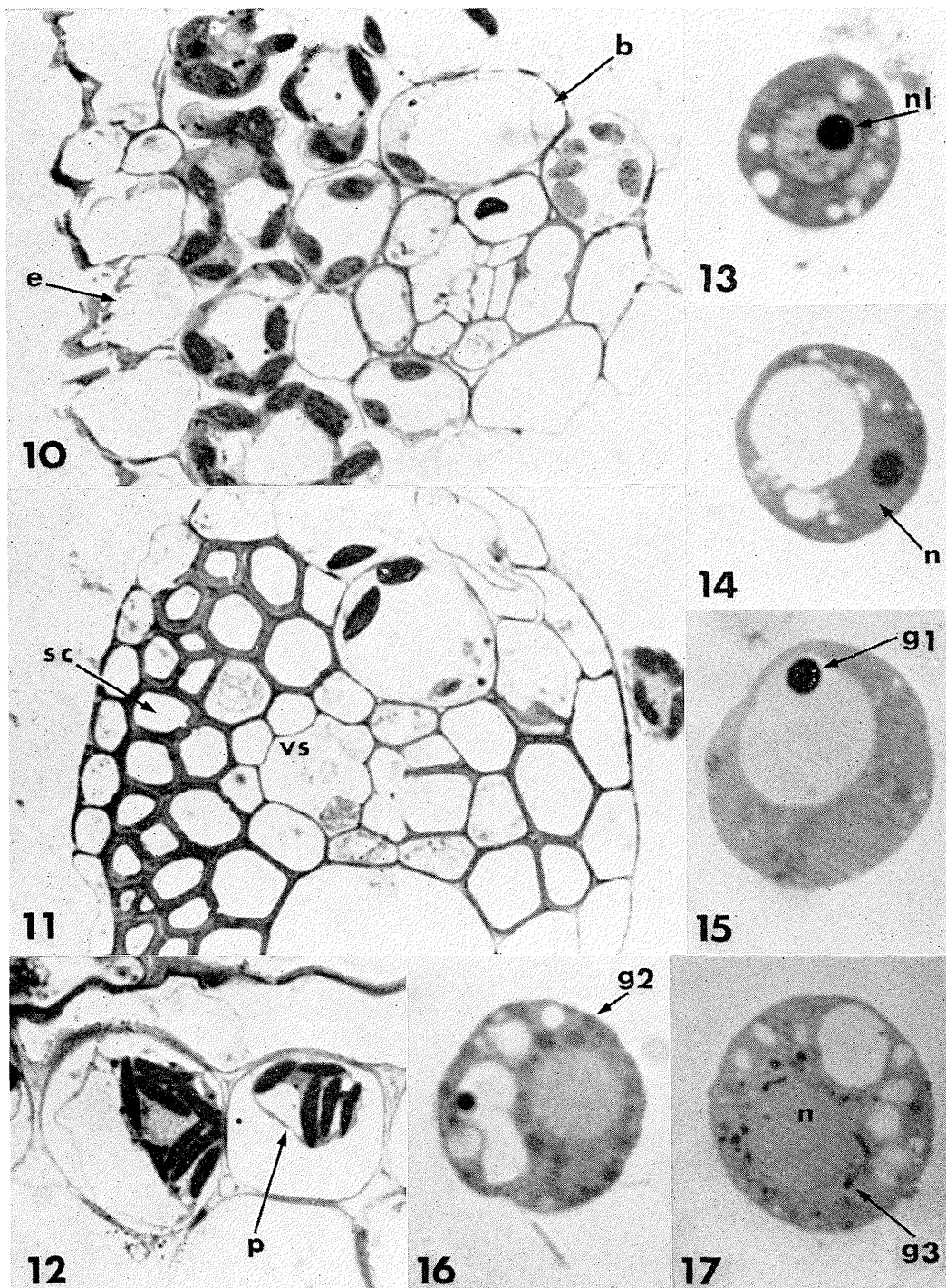
The callus protoplasts are more variable and larger in mean diameter than leaf ones (fig. 1). Such variation seems to be owing to the culture periods and the region from which the experimental materials were obtained. Giant protoplasts having 50 to 75 μm in diameter were extraordinarily seen, as not representing in fig. 1. A large protoplast is shown in fig. 3. In the enzyme suspension, the collapsed protoplasmic materials amorphous in shape were often seen. But it could not be ascertained whether these materials are occurred as a consequence

Key to abbreviations

b: bundle sheath, c: chloroplast, e: epidermal cell, g1: granule of type 1, g2: granule of type 2, g3: granule of type 3, ls: lamellar structure, m: malformed nucleus, n: nucleus, nl: nucleolus, p: plasmalemma, sc: sclerenchymatous cell, v: vacuole, vs: vascular system.

Fig. 2. Unfixed protoplasts liberated from rice callus tissues. An arrow shows a polynuclear like protoplast. ($\times 600$). Fig. 3. A large protoplast of callus ($\times 600$). Fig. 4. Unfixed protoplasts liberated from rice leaves ($\times 600$). Figs. 5 to 9. Semithin sections of leaf protoplasts ($\times 2,350$). Fig. 5: A protoplast having many chloroplasts. Fig. 6: Lamellar structure in chloroplasts. Fig. 7: An uninuclear protoplast. Fig. 8: A quadrinuclear protoplast. Fig. 9: A large protoplast without chloroplasts.





Figs. 10 to 12 Semithin sections of the leaf tissues difficult to be degraded by the enzyme treatment ($\times 1,700$). Fig. 10: Cells near epidermis and vascular system. Fig. 11: Sclerenchymatous cells and vascular system. Fig. 12: Mesophyll cells. Figs. 13 to 17. Semithin sections of callus protoplasts ($\times 2,500$).

of the damage during enzyme treatment or of the aged and dead cells which had existed before treatment.

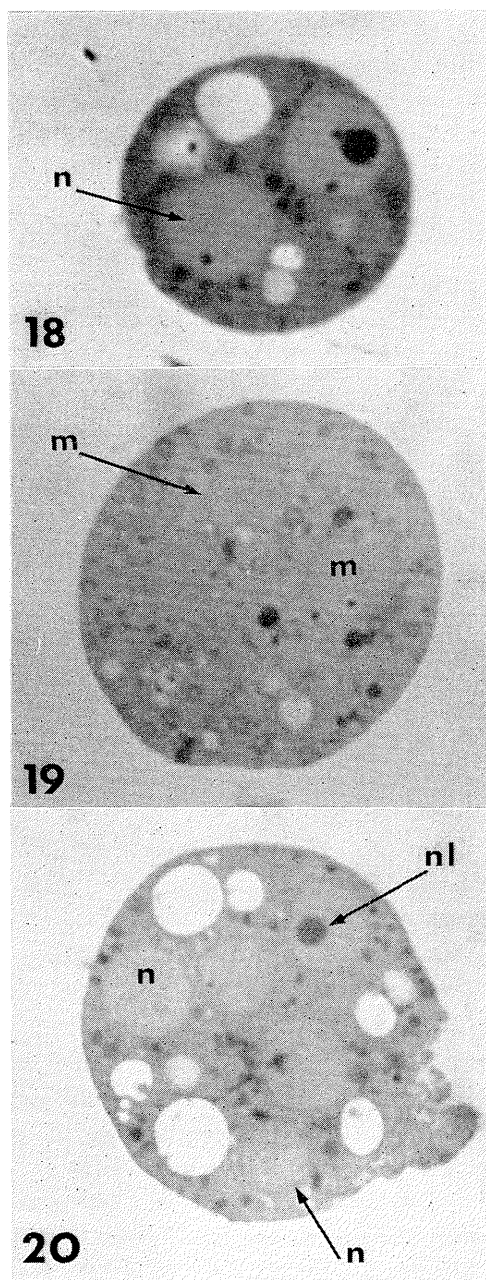
Nucleoli are frequently observed in callus protoplasts (figs. 13, 14, 18 and 20). Nuclei being various

situation are shown, in some nuclei chromatin is condensed (fig. 13) and in others not (fig. 14). There are 3 types of granules in various size in the protoplasts. Fig. 15 shows a large granule, type 1, being seen in the vacuole. Fig. 16 shows granules of round shape, type 2, which present scatteredly in the cytoplasm. Small granules, type 3, are localized in the cytoplasm surrounding the nucleus (fig. 17). Fine structure of these cell organelles is remained to be studied by electron microscopy.

An interesting evidence, which was revealed with the semithin sections of protoplasts produced from rice leaves and callus cultures, is that polynuclear protoplasts were seen frequently. Polynuclear protoplasts are shown in figs. 2, 8, 18, 19 and 20. It is probable that they are induced by spontaneous fusion of protoplasts but not by nuclear division, because of fixation immediately after the short time treatment of enzymes. In the callus protoplasts, it may be considered that polynuclear protoplasts are liberated from polynuclear cells which would have been induced during aseptic culture^{18,21}. While, in the case of leaf protoplasts, it is highly probable that the induction of polynuclear protoplasts is owing to the process of fusion, because polynuclear cells have not been observed in mesophyll tissues of rice yet. Fig. 19 shows irregular form of nuclei, which structure will be future problem to be elucidated.

Root protoplasts have relatively small size of 12.5 μm in mean diameter but large variation (fig. 1). These derived from near root apex are small. But those isolated from the region far from root apex are large. We could find that they occur from the tissues surrounding the vascular system. The root protoplasts have well developed vacuole in most cases, so that thin layer of the cytoplasm is seen along the plasmalemma.

Protoplasts were mostly bursted in hypotonic solution and then their contents flowed out into the media. Therefore, the production of large amount of plant protoplasts is the first step to study the intact state of organelles, that is, nuclei and chloroplasts,



Figs. 18 to 20 Semithin sections of polynuclear protoplasts liberated from rice callus ($\times 2,500$). Fig. 18: A binuclear protoplast. Fig. 19: A protoplast having two malformed nuclei. Fig. 20: A cinquenuclear protoplast.

etc. Rice protoplasts remained alive for one week or more under a cool condition of about 5°C. Accordingly, it is possible for them to be stored and collected for long period. In our experiments of rice protoplasts, it was also ascertained that cellulase R-10 is about five fold in the activity of P-1,500. Further, we tried to degradate the cell wall of various flower cells enzymatically and succeeded in isolation of the petal protoplasts of carnation (*Dianthus superbus*) and *Freesia hybrida*. Such investigation may provide an available method to research the flower pigments.

On the basis of the above information, it may be said that such fascinating problems as transfer of genetic materials or transplanting of cell organelles became to be realizable in rice plant, too. Because some evidences have been already shown in relation to uptake of exogenous DNA²⁴⁾ and bacteria⁴⁾, nuclear fusion²²⁾, plant regeneration^{8,15,26)}, and interspecific hybrids^{9,7)} using several kinds of plant protoplasts. And also plant protoplasts are useful tools to research the action of herbicides²⁾ and DNA isolation²⁵⁾.

The present paper indicates the conditions under which the rice protoplasts are surely produced from the seedlings and callus cultures. In future step, we have to research the circumstances under which they can develop into the callus tissues which ultimately get to rice plantlets. Work is in progress to elucidate the fine structure of rice protoplasts in order to disclose the process of cell-wall regeneration and cell fusion.

SUMMARY

The isolation of rice protoplasts from leaves, roots and callus cultures has been examined following the inoculation in cell wall-degrading enzyme solution. It was achieved within 2 hours at 35°C with the materials cut into small pieces using the mixed solution containing macerozyme, cellulase and mannitol etc. The suitable concentration of enzymes and mannitol to isolate the leaf, root and callus protoplasts was ascertained. The size of protoplasts was measured in their diameter and the frequency distribution was acquired. The size was large in the callus protoplasts and small in the leaf ones. Structure of the protoplasts was investigated with the semithin sections by

light microscope. Nature of nuclei, nucleoli and lamellar structure in chloroplasts are illustrated. The spontaneous fusion of protoplasts was assumed from the presence of polynuclear protoplasts. In addition, the protoplast isolation from flower petals was performed successfully using carnation and *Freesia*.

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〔和 文 摘 要〕

酵素処理によるイネの葉およびカルスからの原形質体の分離

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イネ幼苗の葉と根および無菌培養カルスから、原形質体を分離する方法を検討した。こまかく切った試料を *macerozyme*・*cellulase*・*mannitol* などの混液に 35°C 条件で 2 時間処理し、葉・根およびカルス起源の原形質体の作成に成功した。各原形質体の分離に必要な条件をしらべ、形成された原形質体の大きさの頻度分布を求めた。分離し遠心処理により集められた原形質体を、グルタルアルデヒドとオスミック酸で固定し、エポン樹脂に包埋し準超薄切片を作成した。トルイジンブルー・フクシンメチレンブルー・PAS トルイジンブルーなどで染色し、光学顕微鏡で細胞内顆粒体を観察した。

葉起源原形質体の作成には、適当な令の幼苗を使用する必要がある、カルス起源の原形質体の作成には、移植後日数とカルスの部位を選ぶことが必要であることを明らかにした。葉の維管束鞘および表皮細胞の細胞壁は、酵素処理により分解されにくく、葉緑体をもつた原形質体の多くは、葉肉細胞から生じたものと考えられる。葉起源およびカルス起源の原形質体のなかに、多くの多核性のものを見ることができた。カルスの場合すでに多核細胞の存在することが知られているが、イネの葉には多核細胞が認められていないので、葉起源の原形質体は、短時間の酵素処理中に自然融合することによつて、多核化したものと考えられる。またカーネーションやフリージャの花弁細胞からも、原形質体を作成し得ることを示した。