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RICE (*ORYZA SATIVA* L.) PROTOPLAST[1,2]

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Abstract

A high yield of protoplast can be obtained from young leaf blades of various rice species e.g. *Oryza sativa* L. var. Taichung No. 65, *O. sativa* L. var. Taichung No. 1, and *O. sativa* L. var. Tainan No. 5 by using an enzyme combination (4% "Onozuka" cellulase R-10 and 2% pectinase). The isolated protoplasts were mostly spherical in shape. The viability of the protoplast remained alive for two weeks or more under 4°C incubation. Fusion has been observed when the washed protoplasts were suspended in lysozyme or PEG 6000 solution and its frequency was high. The neutral red staining technique is a good marker for heteroplasmic fusion of protoplasts.

Introduction

The enzymatic isolation of higher plant protoplasts in large numbers was first described by Cocking (1960). Further development of this technique and the available commercial enzymes made it possible to isolate protoplasts from various plant tissues such as root tips (Power *et al.*, 1970), cotyledons (Cocking, 1962), coleoptiles (Ruesink and Thimann, 1966), vegetable petals (Tseng *et al.*, 1975), mesophyll leaves (Takebe *et al.*, 1968), from callus tissues (keller *et al.*, 1970), cell suspension cultures (Schenk and Hildebrandt, 1969) and pollen tetrads (Bhojwani and Cocking, 1972). The fusion of isolated protoplasts from higher plants by using fusion-inducing agent such as sodium nitrate was first reported by Power *et al.* (1970). Since that time a number of reports have been published on the intra- and interspecific fusions of higher plant protoplasts (Cocking, 1972; Kao and Michayluk, 1974).

Recently, protoplasts of higher plants have come into the limelight as a new experimental system, because of their various unique characteristics which can not be seen in ordinary cells covered with cell walls. For instance, some characters of other plants and microorganisms can be introduced into protoplasts by incorporation of hereditary substances such as deoxyribonucleic acid, ribo-

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nucleic acid and nucleoprotein particles (Aoki and Takebe, 1969; Ohyama *et al.*, 1972; Takebe and Otsuki, 1969). Such characteristics of protoplasts are not only interesting from the viewpoints of cell biology and genetic but also able to be used in the area of crop breeding (Carlson *et al.*, 1972).

Studies on rice protoplasts are still meager, except the enzymatic isolation of rice protoplasts from leaves, roots and callus mass was reported by some workers (Maeda *et al.*, 1974 and Liu, 1975), and the fusion of isolated protoplasts from rice callus by Harn (1973). In our previous report (Tseng *et al.*, 1975), the isolation of rice protoplast from young leaves and callus was demonstrated by using a cell wall degrading enzyme mixture. However, since the optimal conditions for protoplast isolation, such as the age of plants and the combination of enzymes were not carefully controlled, the yield of protoplast may not be the highest obtainable one. This paper presents the proper conditions for rice protoplast isolation, and the fusion of isolated protoplast either intra- or interspecific fusion are also discussed.

Materials and Methods

Protoplast isolation

Protoplasts were isolated from leaves (young leaf blades) of greenhouse grown rice plants (e.g. *Oryza sativa* L. var. Taichung No. 65, No. 5 and *Oryza sativa* L. var Taichung No. 1). The seeds were soaked in water at 30°C for 3 days, changing water daily, and then seeded on plastic trays ($6\times20\times25\,cm^3$) which contained sterile soil with fertilizer in it. The plants were cultured at 30°C and illuminated under discontinus fluorescence light (9hr in dark and 15hr in light; 2000–3000 lux). After several weeks, the young leaf blades were excised and ready for protoplast isolation. Sample of 0.8 g fresh weight leaves was cut into narrow, longitudinal shreds using a scalpel, and the leaf pieces were immersed in an 100 ml of Erlenmeyer flask which contained 10 ml of 0.6 M sucrose solution. It was incubated and shaked in a Dufnoff Metabolic Shaking Incubator (150 r.p.m.) at 37°C for 20 min. After pre-incubation, the reaction fluid was then decanted and replaced with a 8 ml of various enzyme mixture contained 4% "Onozuka" cellulase R-10 or 4% Meicelase with 2% pectinase in 0.6 M sucrose solution pH 5.6; or 4% of "Onozuka" cellulase R-10 with 2% Macerozyme in the same sucrose concentration (pH 5.6) as well as another enzyme mixtures. Thereafter, the pieces of rice leaves were incubated in the enzyme mixture at 37°C for $1\frac{1}{2}$ hr and shaken as described above. After incubation the mixture was set at 37°C for another 20 min, the suspension of isolated rice protoplasts thus obtained was passed through four layers of Nylon cloth to remove cell debris. The filtrate which contained

protoplasts was kept for 10 min to allow any small debris to settle, and then the protoplasts which were floating up and being concentrated on the surface were transferred to another comparable test tube which contained 9 ml of 0.6 M sucrose. The procedures were repeated once. These protoplasts were referred to as "twice-washed protoplasts". The washing procedure is to dilute out the enzymes and toxic compounds from rice tissue. The isolated protoplasts were observed under a light microscope and number of protoplasts was counted by a Haemacytometer.

Protoplast fusion

Approximately 150 μl of the protoplast suspension, *Oryza sativa* L. var. Taichung No. 65, was pipetted onto a depression slide, prepared by pressing a 2-layer thick parafilm ring (dia. 1 cm) onto warmed microscope slide. A bit amount of silicone grease was applied to the slide just inside the ring to prevent liquid from being drawn under the parafilm by capillarity. After the protoplasts had settled, an equal volume of fusion-inducing agent prepared in 0.6 M sucrose was slowly added to the depression slide and then it was incubated at room temperature for 10 min before covering with glass slip. Fusion of the protoplasts was monitored under light microscope. The protoplast fusion was also performed by using different varieties of rice plants such as *Oryza sativa* L. var. Taichung No. 65 and *O. sativa* L. var. Taichung No. 1. That is, one of the isolated protoplasts (3 ml) was transferred to a test tube containing 0.6 M sucrose with 0.01% neutral red pH 7.0. After incubation at room temperature for 40 min, protoplasts which stained with the vital dye (Fig. 7) were served for interspecific fusion. Equal volume of two different kinds of protoplasts were mixed in 1:1 (v/v) proportion and placed in a depression slide, after setting the fusion-inducing agent was added and examined as described above. Polyethylene glycol, lysozyme, sodium nitrate, and Tween 20 were served as fusion-inducing agents. The frequency of protoplast fusion was expressed as the percentage of homoplasmic fusion (intra-specific fusion) or heteroplasmic fusion (interspecific fusion) in the total number of free protoplasts and fused protoplasts.

Chemicals

Cellulase "Onozuka" R-10 and Macerozyme R-10 were purchased from Kinki, Yakult Mfg, Co. Ltd., 8-21, Shingikancho, Nistinomiya, Japan; Meicelase was obtained from Sigma Chemical Company. Polyethylene glycol (m. w. 6000) was purchased from Woko Pure Chemical Industries Ltd, and Tween 20 from Altas Chemical Industries Ing., Washington, Delaware, U. S. A.

Results

In the current study, the suitable conditions for the rice protoplast isolation were carefully examined. The effects of various enzymatic combinations on released protoplasts from rice leaves were presented in Table 1. Results showed that one of the enzyme combination, 4% cellulase "Onozuka" R-10 and 2% pectinase used as an enzyme mixture for protoplast isolation, exhibited a high yield of protoplasts as compared with the other enzyme mixtures. Pectolytic enzymes such as Macerozyme R-10 and pectinase seemed to have no appreciable effect on isolation of protoplasts from rice leaves, also, the enzyme mixtures with or without dextran sulfate have no significant effect on the yield of protoplast released. The isolated protoplasts were mostly spherical in shape (Figs. 1, 2, 3 and 4). The diameter of isolated protoplasts was not estimated, however, from the figures it was clearly showed that their morphological characteristics were similar but sometimes a few giant protoplasts were found.

Table 1. *Effects of various enzyme combinations on release of protoplasts from young leaf blades of rice*(1)

Enzqme mixture(2)	Yield of protoplasts/g. fresh weight(3)
4% cellulase "Onozuka" R-10 + 2% Macerozyme R-10 + 0.1% dextran sulfate	6.8×10^5
4% cellulase "Onozuka" R-10 + 2% Macerozyme R-10	6.4×10^5
2% Myrothecium cellulase + 2% pectinase	1.0×10^2
4% cellulase "Onozuka" R-10 + 2% pectinase	7.5×10^7
4% Meicelase + 2% pectinase	3.5×10^4

(1) Young leaf blades of 6 weeks old rice plants, ***Oryza sativa*** L. var. Taichung No. 65, were used as tested materials.
(2) All of the enzyme mixtures were dissoloved in 0.6 M sucrose pH 5.6. Myrothecium cellulase was prepared as previous described (Tseng ***et al.***, 1975).
(3) Number of protoplasts was counted by a Haemocytometer.

Age of rice plant played a major factor for protoplast isolation. Although viable protoplasts could be isolated from various stages of rice leaves. However, in the current study, it showed that the young leaves of 4–8 weeks old rice were more suitable materials for protoplast isolation. No protoplasts were

obtained when 16 weeks old rice plants were served as tested materials (Table 2). We also found that the protoplasts were scarcely produced at the time of one week old seedling.

Table 2. *Effects of various rice species and age of material used on the isolation of rice protoplasts.* Rice protoplasts were isolated from various age of rice young leaf blades. The enzyme mixture used for the protoplast isolation was 4% cellulase "Onozuka" R-10 and 2% pectinase in 0.6 M sucrose pH 5.6.

Species	Yield of protoplasts/g. fresh weight Age (weeks)					
	2	4	6	8	12	16
Oryza sativa L. var. Taichung No. 1	0.6×10^6	1.18×10^7	3.2×10^7	8.4×10^7	1.2×10^4	—
Oryza sativa L. var. Tainan No. 5	0.1×10^6	2.4×10^7	1.8×10^7	9.0×10^7	1.2×10^4	—
Oryza sativa L. var. Taichung No. 65	5.0×10^5	3.2×10^7	3.3×10^7	7.5×10^7	5.9×10^5	—

The stability of the isolated protoplasts in 0.6 M sucrose under various temperature conditions was illustrated in Table 3. Apparently when the isolated protoplasts preserved at 4°C, it was observed that most of the protoplasts were still survial after 4 days incubation. On the contrary, the viability and number of the protoplasts drastically changed when it was preserved at the temperature of 25°C and 30°C, and it showed the protoplast suspension brought bacterial contamination after prolonged incubation. No viable protoplasts were found after 4 days incubation at 30°C.

Table 3. *The viability of isolated rice protoplasts under various preservation time and temperature treatments*(1)

Time of preservation (day)	No. of survival protoplasts/ml(2)		
	4°C	25°C	30°C
1	1.04×10^7	2.52×10^6	3.0×10^4
2	2.40×10^7	4.0×10^4	1.0×10^3
3	2.80×10^7	3.8×10^4	3.0×10^2
4	5.92×10^6	1.2×10^4	—
6	1.01×10^6	0.8×10^3	—
14	5.00×10^4	—	—

(1) Young leaf blades of 6 weeks old rice plant, *Oryza sativa* L. var. Taichung No. 65, were used as tested materials.
(2) Number of protoplasts was counted by a Haemocytometer.

The fusion of rice protoplast has been carried out by using various fusion-inducing agents. Results from Table 4 revealed that homoplasmic fusion of isolated protoplast was achieved by using various lysozyme concentrations, but a high concentration of lysozyme (0.5%) led to destruction of the protoplasts. We have obtained the best fusion results with 0.125% of lyozyme. The protoplast aggregates often involved agglutination of many protoplasts (Fig. 6), but some aggregates consisted of only two protoplasts (Fig. 5).

When PEG 6000 was served as fusion agent, in most of cases, we found protoplasts instantly shrunk, it might be due to the fact of sudden and strong dehydration. However, in less than a minute the protoplasts have aggregated and fusion occurred within a few minutes. In Table 4 indicates that higher frequency of homoplasmic fusion (46.5%) can be obtained with 15% PEG.

Table 4. *Influence of various fusion-inducing agents on rice leaves protoplast fusion*(1)

Agent(2)	Concentration in treating solution (%)	Time for protoplast aggregation(3) (min)	Frequency(4) (%)
Lysozyme	0.500	—	—
Lysozqme	0.250	20	18.93
Lysozyme	0.125	15	24.94
Lysozyme	0.025	18	20.17
Lysozyme	0.013	20	18.38
PEG 6000	15% plus 7mM $CaCl_2$	2	46.5
PEG 6000	5% plus 7mM $CaCl_2$	7	6.6
Sodium nitrate	4.25	—	—
Sodium nitrate	2.12	10	0.5
Tween 20	40	40	0.01
Tween 20	35	20	0.3

(1) Young leaf blades of 6 weeks old rice plants, *Oryza sativa* L. Taichung No. 65, wereused as tested materials.
(2) All of the fusion-inducing agents were dissolved in 0.6 M sucrose before use.
(3) Time indicates the protoplast aggregates after adding fusion-inducing agent.
(4) Frequency represents the percentage of homoplasmic fusion of two protoplasts.

Heteroplasmic fusion of isolated rice protoplasts was performed by using neutral red staining technique. For example, *Oryza sativa* L. var. Taichung No. 1 was stained with neutral red after isolation. Figure 7 showed all of the protoplasts retained the red color when it was incubated with the dye at room temperature for 40 min. Thus, the protoplasts which stained with the dye were used for heteroplasmic fusion. Equal volume of two different sources of protoplasts were mixed and placed in a depression slide, after setting the fusion-inducing agent, PEG 6000, was added and examined under light microscope.

The results from Table 5 illustrated that several fusion patterns were observed, they were either homoplasmic or heteroplasmic protoplast fusion and sometimes the protoplast aggregates involved more than two protoplasts. The frequency of heteroplasmic fusion (13.5%) was much less as compared with homoplasmic fusion (46.5%) which appeared in Table 4.

Table 5. *Fusion of rice protoplasts for* ***Oryza sativa*** *L. var. Taichung No. 65 and* ***Oryza sativa*** *L. var. Taichung No. 1 by using polyethylene glycol 600*[1]

Fusion pattern[2]	Time for protoplasts aggregation[3] (min)	Frequency[4] (%)
Homoplasmic fusion A ○○	8	12.5
Heteroplasmic fusion ○●	6	13.5
Homoplasmic fusion B ●●	3	21.0

(1) Six weeks old young leaf blades were used as tested materials. The fusion-inducing agent consists of 15% polyethlene glycol 6000 and 7 mM $CaCl_2$ in 0.6 M sucrose.
(2) The isolated protoplasts from *Oryza sativa* L. var. Taichung No. 1 were stained with neutral red before use.
●……stained protoplast
○……unstained protoplast
(3) Same as Table 4 described.
(4) Frequency represents the % of homoplasmic or heteroplasmic fusion of two protoplasts.

Discussion

In the previous report we have demonstrated that using an enzyme mixture of cellulase and Macerozyme was able to isolate protoplasts from mesophyll and callus tissues of many crop plants (Tseng *et al.*, 1975). However, the yield of the isolated protoplasts does not represent the best obtainable results for individual species, since the proper age of the materials and the condition of optimal enzyme mixture for protoplast isolation are scarcely controlled.

The sucess of isolation of vital protoplasts from higher plants mainly depends on several parameters such as age and physiological condition of the tested plant, nature and concentration of plasmolyticum, concentration of enzyme, temperature, pH, time of incubation, and shaking or not shaking during incubation (Cocking, 1972). One must investigate systematically the plant tissue in relation to the optimal condition for protoplast released. In the current study, attempts were made to search suitable conditions for isolation of vital protoplast from rice young leaf blades. Base on our experimental

conditions, the effects of different enzymatic combination on the release of protoplasts from rice leaves were demonstrated. An enzyme mixture which consists of 4% cellulase "Onozuka" R-10 and 2% pectinase in 0.6 M sucrose pH 5.6 exhibited a higher yield of protoplast as compared with the other enzyme cominations; 4-8 weeks old of rice young leaves are more suitable for the protoplast isolation, the protoplasts are scarcely produced at the age of rice plant older than 12 weeks or younger than one week after seed germination. However, Maedal *et al.*, (1974) reported that a few protoplasts of rice seedlings were produced at the age older than 17 days after seed germination. The seedlings which they used for the protoplast isolation were cultured without nutrients at 30°C under continueous fluorescence light, while, in our case, the greenhous grown rice plants are served for the protoplast isolation. The differences of our results could not be ascertained, it may be due to the fact of different physiological conditions of rice plants. The viability of the isolated protoplasts in most of the cases remains alive for two weeks or more under a cool condition of 4°C. Thus, one can isolate the protoplasts and store them in a cool condition until use.

The protoplast fusion of various higher plants were reported by several workers (Power *et al.*, 1970; Gamborg, *et al.*, 1972; Eriksson 1970). Harn (1973) reported that the fusion took place immediately after the washed rice protoplasts transfer to sodium nitrate, but the frequency of fusion was low. In this study, we found that both lysozyme and PEG 6000 provided better fusion rate of rice protoplasts as compared with the other agents such as sodium nitrate and Tween 20.

Heteroplasmic fusion of rice protoplasts e.g. *Oryza sativa* L. Taichung No. 65 and *O. sativa* L. var. Taichung No. 1 has been achieved by using PEG 6000 as fusion agent. However, early in this work, we had difficulty to identify the fusion aggregates (heteroplasmic fusion), which exhibited no morphological differences of the isolated protoplasts. Thus, a method of neutral red staining technique for protoplast fusion were developed from this laboratory. That is one of the best techniques for heteroplasmic fusion of protoplasts.

In the rice protoplast research, it is essential that the isolated protoplasts should retain the capability of regenerating normal plants. Work in this area is in progress to culture the isolated protoplasts from various rice species.

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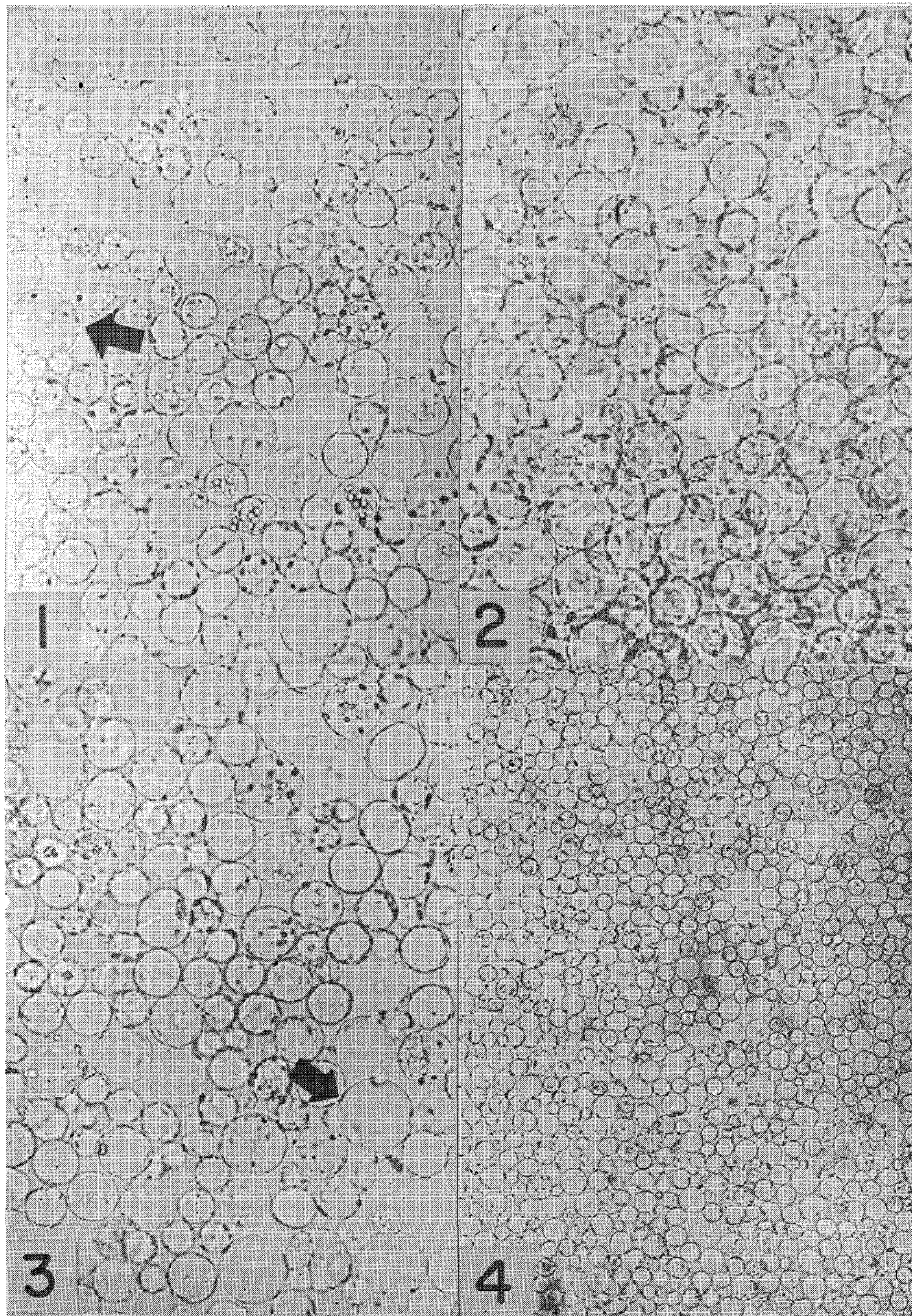
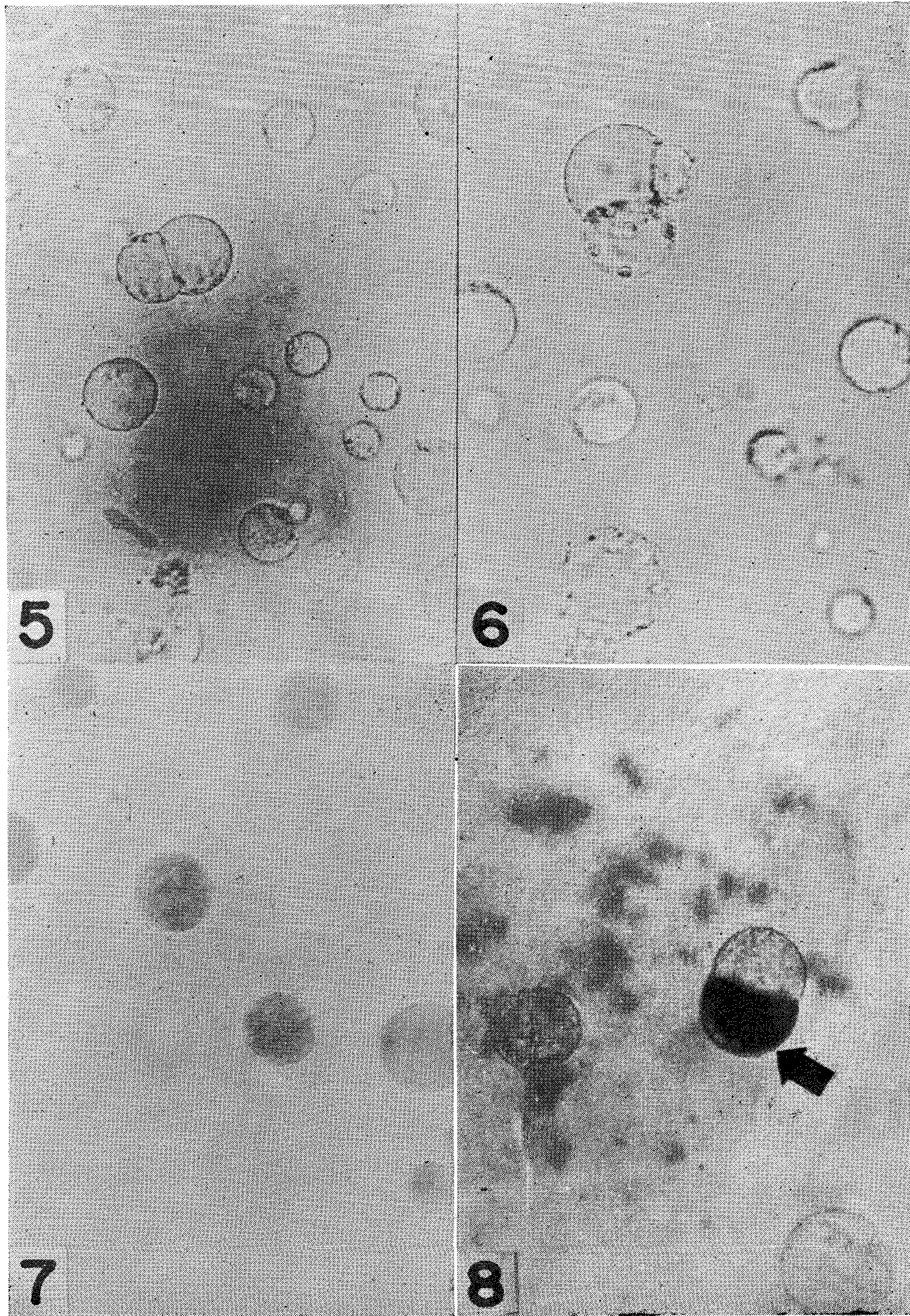


Figs. 1-4. Protoplasts isolated from 6 weeks old young leaf blades of various rice species.
1. Protoplasts of ***Oryza sativa*** L. var. Tainan No. 5 (×915)
2. Protoplasts of ***Oryza sativa*** L. var. Taichung No. 1 (×915)
3 and 4. Protoplasts of ***Oryza sativa*** L. var. Taichung No. 65 (×915, ×146)

All of the photos were taken under a light microscope. Arrow represents a giant **protoplast.**



Figs. 5-8. Aggregation and fusion of rice protoplasts. 5. Lysozyme-induced aggregates (two protoplasts) of *Oryza sativa* L. var. Taichung No. 65 protoplasts (×1464). 6. Lysozyme-induced aggregates (three protoplasts) of *Oryza sativa* L. var. Taichung No. 65 protoplasts (×1500). 7. Neutral red stained protoplasts of *Oryza sativa* L. var. Taichung No. 1 (×1500). 8. Lysozyme-induced heteroplasmic fusion of protoplasts of *Oryza sativa* L. var. Taichung No. 1 and *Oryza sativa* L. var. Taichung No. 65 (×1500). Arrow indicates a neutral red stained protoplast.

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水稻原生質體

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利用一種果膠質和纖維分解酵素混合液（"Onozuka" cellulase R-10 和 Pectinase）去處理不同水稻品種嫩葉，例如臺中65號，臺中一號和臺南 5 號，可得大量原生質體。被分離出之原生質體一般呈圓形，將它放在 4°C 培養時，雖歷經兩星期之久，原生質體仍保持其活性。若將洗過之原生質體懸浮在 Lysozyme 和 PEG 6,000 之溶液中，可促使其發生融合之現象，且融合率頗高。在做不同種之水稻原生質體融合時，可藉 Neutral red 之染色方法區別其品種。