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SHORT COMMUNICATION

ISOLATION OF PROTOPLASTS FROM CROP PLANTS[1,2]

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The use of plant protoplasts has been proposed as a unique tool for studying such fundamental problems of plant cells as division, growth, differentiation, morphogenesis, virus infection and somatic hybridization (Schenk, 1968; Schenk and Hildebrandt, 1968). Enzymatic isolation of living protoplasts from higher plants is considered to be first step toward this goal.

Cocking (1960) first succeeded in preparing protoplasts from tomato root tissue by digestion with cellulase from *Myrothecium verrucaria.* The procedure was later extended by Ruesink and Thimann (1966), again using a concentrated *Myrothecium verrucaria* cellulase, to the isolation of protoplasts from leaf coleoptile and callus tissue. In 1969, cellulase "Onozuka" p 1500, a crude cellulase preparation from *Trichoderma viride* which had then become commercially available in Japan, was used for the isolation of protoplasts from cell suspensions of *Haplopappus gracilis* (Eriksson and Jonasson, 1969). The commercial availability of such cellulases greatly stimulated the work on protoplast isolation. More recently, the use of commercial cellulases has been extended to the isolation of protoplasts from a wide range of plant cells in liquid culture by Schenk and Hilderbrandt (1969). Otsuki and Takebe (1969) obtained mesophyll protoplasts from many herbaceous species of angiosperms by using a mixture of commercial cellulase and pectinase. The successful isolation of a wide range of plant protoplasts encouraged us to extend the application of these techniques to other crop plants, particularly the plants which are grown in this area. This report presents the results of enzymatic preparation of protoplasts from diverse species of crop plants.

Culture of *Myrothecium verrucaria,* obtained from our laboratory, was used for preparation of *Myrothecium* cellulase. The enzyme was isolated and

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partially purified by ammonium sulfate saturation and gel filtration chromatography (Ruesink and Thimann, 1966). The commercially available enzymes, such as cellulase "Onozuka" R-10, Macerozyme R-10 (Kinki, Yakult Mfg. Co. LTD., 8-21, Shingikancho, Nishinomiya, Japan) and Pectinase (Sigma Chemical Company, P. O. Box 14508, St. Louis, Mo. 63178, U. S. A.) without purification were also used for this purpose.

Crop plants were selected mainly on the basis of convenience in obtaining the materials. Plants were either grown in a growth chamber or purchased from markets as whole plants. Both expanding leaves and mature leaves with a fresh appearance were used. No special considerations were made such as age and physiological condition of the plants. The callus tissues used in these studies were induced from various kinds of seeds except that rice callus tissues were induced from pollen and potato from tuber. They were routinely grown on agar media (Murashige and Skoog, 1962), and were transferred to fresh culture two weeks before they were used.

The procedure for the isolation of protoplasts from mesophyll tissues was as follow. With the aid of forceps, the lower epidermis of mesophyll tissue was peeled from excised leaves and the stripped leaves were then cut into small pieces (ca. 4×4 mm). One gram of the tissues was placed in 10 ml of 0.6 M sucrose solution in a 100 ml Erlenmeyer flask. The flask was then incubated in a water bath at 35°C and shaken by hand at 5 min intervals. After 20 min preincubation, the reaction fluid was removed by decantation and was replaced by a 10 ml of enzyme mixture. The enzyme mixture was either a mixture of 4% (W/V) cellulase "Onozuka" R-10, 2% (W/V) Macerozyme R-10 and 1% dextran sulfate in 0.6 M sucrose solution pH 5.8 or a mixture of 4% (W/V) *Myrothecium* cellulase and 2% (W/V) pectinase (Sigma) with 1% (W/V) dextran sulfate in the same sucrose concentration (pH 5.8). Thereafter, the pieces of mesophyll tissues were incubated in the enzyme mixture at 35°C for a certain peroid of time and shaken occasionally. The suspension of isolated protoplast thus obtained was passed through three layers of Nylon cloth to remove cell clumps and large cell debris. The isolated protoplasts were then washed twice with 0.6 M sucrose in order to be free from enzyme contamination. Number of protoplasts was measured by a Haemacytometer under a light microscope.

Table 1 lists the results obtained from 20 species of crop plants. Apparently, when a mixture of cellulase "Onozuka" and Macerozyme were used, they were able to release more of mesophyll protoplasts from the leaves of most species tested as compared with the enzyme mixture which consisted of *Myrothecium* cellulase and pectinase. No protoplasts but a lot of single cells were released from mesophyll tissues of soybean, mungbean and chinese leek when they were

Table 1. *Isolation of protoplasts from mesophyll tissues of crop plants.* (*a*)

Species	Cellulase "Onzuka"+Macerozyme (b)		Myrothecium cellulase+Pectinase (c)	
	Time of incubation (hr)	Protoplasts obtained (No./ml)	Time of incubation (hr)	Protoplasts obtained (No./ml) (d)
Glycine max Merr.	2	—	2	—
Phasealus vulgaris L.	2	—	2	—
Amaranthus palmeri L.	2	3.0×10^4	2	—
Brassica chinensis L.	2	0.3×10^4	2	—
Brassica campestris L.	2	2.2×10^4	2	—
Chrysanthemum coronarium L.	2	3.4×10^4	2	—
Colocasia esculenta (L.) Schott	2	1.0×10^4	2	—
Allium cepa L.	2	0.9×10^4	2	—
Brassica juncea Cosson	2	3.4×10^4	2	0.2×10^2
Raphanus sativus L.	2	1.8×10^4	2	1.0×10^2
Allium fistulosum L.	2	—	2	—
Apium graveolens L.	2	4.0×10^4	2	—
Lycopersicum esculentum Mill	2	3.6×10^4	2	—
Solanum tuberosum L.	2	4.3×10^4	2	—
Bambusa sp.	2	9.6×10^4	2	—
Oryza sativa L.	2	6.8×10^4	2	—
Saccharum officinarum L.	2	4.0×10^4	2	0.1×10^2
Brassica pekinensis Rupr.	3	1.1×10^4	3	—
Spinacia oleracea L.	4	8.0×10^4	4	—
Brassica oleracea L. Var. *botrytis* L.	4	2.0×10^4	4	—

(a) Upper epidermis was peeled off except tissues of bamboo, rice and sugar cane were cut into small pieces (ca. 4×4 mm). One gram of the tissue was treated with the enzyme mixture and incubated at 35°C for a certain period of time.
(b) Enzyme mixture consisted of 4% (W/V) cellulase "Onozuka" R-10, 2% (W/V) Macerozyme R-10 and 1% (W/V) dextran sulfate in 0.6 M sucrose (pH 5.8).
(c) Enzyme mixture contained 4% (W/V) Myrothecium cellulase and 2% (W/V) pectinase in 0.6 M sucrose with 0.01% (W/V) dextran sulfate pH 5.8.
(d) Number of protoplasts was measured by Haemacytometer under light microscope.

treated with either enzyme mixtures. It may be due to the fact that these tissues contained higher amount of calcium which inhibits pectinase activity or due to some other unknown reasons. These results indicate that the yield of protoplasts by this method varied somewhat depending upon individual species.

Microscopic examination showed that in the majority of the isolated mesophyll protoplasts, the chloroplasts were regularly arranged around a central vacuole (Figs. 1 and 2). Protoplasts were also isolated from different parts of tested plants using the same system as described above (Figs. 3, 4, 5 and 6). The stability of isolated protoplasts varies with plant species. Some of them readily survived overnight, while the other often survived a few days or more.

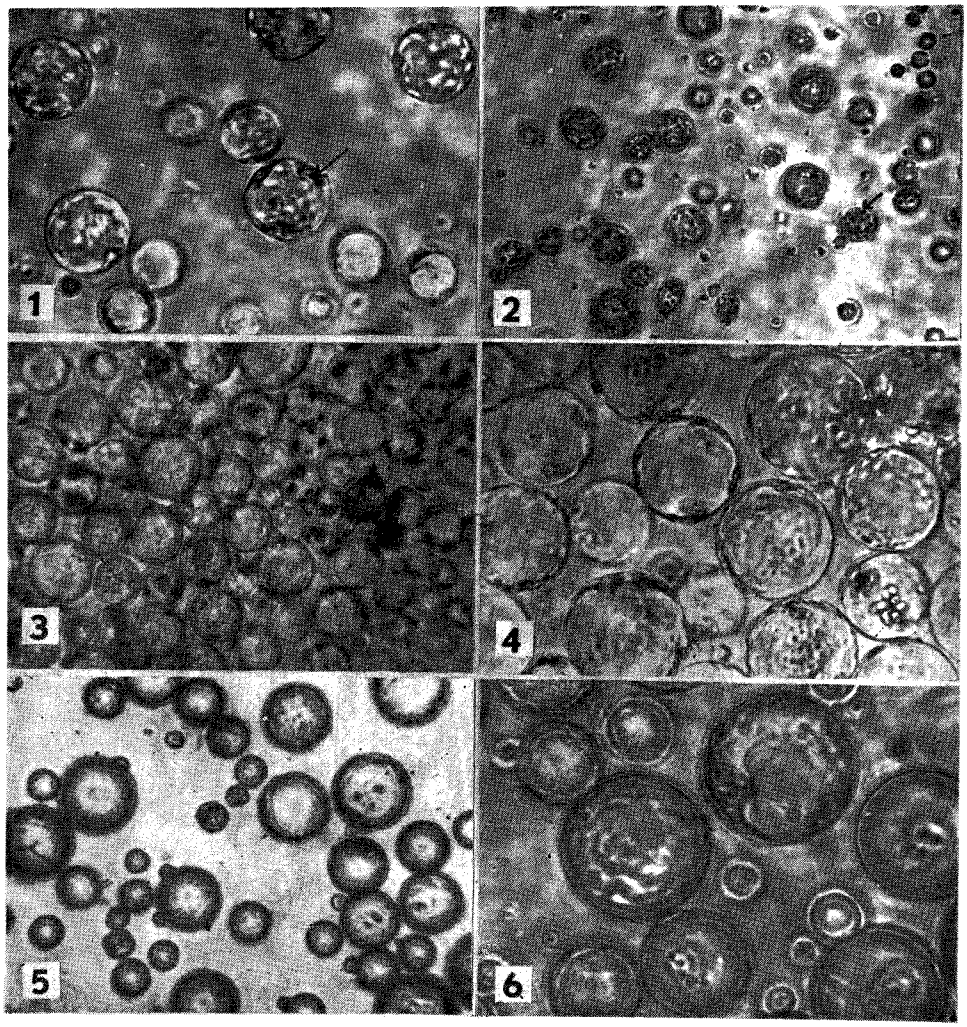
The callus tissues used in these studies were routinely grown on agar media and were transferred to fresh media two weeks before use. One gram of callus was suspended in 6 ml of an enzyme mixture which consisted of 5% (W/V) cellulase "Onozuka", 2% (W/V) Macerozyme and 0.01% (W/V) dextran sulfate in 0.6 M sucrose, pH 5.4. The reaction medium was incubated in 25 ml flask at 37°C for 5 hr and occasionally shaken by hand in order to free protoplasts. Viable protoplasts were obtained from all of 6 species of callus routinely grown in our laboratory (Table 2). The isolated protoplasts usually show vigorous cyclosis. The stability of isolated protoplasts varried greatly between species.

Several methods have been worked out for the isolation of viable protoplasts from various kinds of plant tissues (Cocking, 1965; Pinta, 1969; Ruesink and Thimann, 1966). The present investigation showed that our method, using an enzyme mixture of cellulase "Onozuka" and Macerozyme is a better formula for the isolation of protoplasts from mesophyll and callus tissues of many

Table 2. *Isolation of protoplasts from callus tissues of 6 species of crop plants*

Callus tissues were induced from various seeds except rice from pollen and potato from tuber. One gram of callus tissues was treated with an enzyme mixture containing 5% (W/V) cellulase "Onozuka" R-10, 2% (W/V) Macerozyme and 0.01% (W/V) dextran sulfate in 0.6 M sucrose (pH 5.4) and then incubated at 37°C for five hours. Number of protoplasts was measured by Haemacytometer under a light microscope.

Species	Time of incubation (hr)	No. of released protoplasts (ml)
Glycine max Merr.	5	3.3×10^4
Phaseolus vulgaris L. Var. kidney bean	5	0.6×10^4
Phasealus vulgaris L.	5	1.2×10^4
Nicotiana tabacina L.	5	0.5×10^4
Solanum tuberosum L.	5	5.0×10^4
Oryza sativa L.	5	2.3×10^4



Figs. 1-6. Protoplasts isolated from crop plants. 1. Mesophyll protoplasts of chinese cabbage (***Brasssica pekinensis***) (×1200). 2. Protoplasts of bamboo (***Bambusa*** sp.) young leaves (×720). 3 & 4. Protoplasts of rice (***Oryza sativa***) young leaves (×440, ×1040). 5 & 6. Protoplasts obtained from petioles of chinese cabbage (×350, ×700). All of the pictures were taken under phase contrast microscope. Arrow indicates chloroplast.

crop plants as compared with the other enzyme combination. However, it should be pointed out that the results of this study do not necessarily represent the best obtainable results for individual species, since the proper age and physiological state of the materials are scarcely controlled during the course of work. On the other hand, the conditions of enzyme mixture optimal for isolation of protoplasts may be diverse amony species. Therefore, more detailed examination in this respect of individual species should lead to improve in the yield of protoplasts.

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農作物原生質體的分離

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使用一種纖維分解酵素和果膠質分解酵素混合液，去處理各種不同植物組織時，已成功的得到活的原生質體。在這些被試的二十種不同農作物當中，大部份皆會產生大量的原生質體，但是大豆綠豆和韮菜只能產生單細胞和少量的原生質體。