OBSERVATIONS ON PROTOPLASTS OF CHINESE CABBAGE AND CHINESE CHIVE

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Abstract: Enzymatic isolation of protoplasts from young tissues of Chinese cabbage (*Brassica pekinensis* Rupr.) and Chinese chive (*Allium odorum* L.) has been studied. Different osmotica show variability in quantities of protoplasts. Chinese cabbage gives a good yield of protoplasts (10^8 cells/g tissue), but Chinese chive is poor (10^6 cell/g tissue). Spontaneous fusion of protoplasts occurs and its frequency increases with the prolongation of time. Induced fusion of protoplasts by PEG enhances the fusion frequency drastically (50%-70%). Vital staining with neutral red distinguishes between homokaryotic and heterokayrotic fusion. No heterokaryotic fusion occurring between protoplasts from these plants has been observed.

INTRODUCTION

In the last few years, studies on protoplasts have progressed tremendously. The sources of plant materials have come from leaves, stems, roots and single cells (Cocking, 1960; Nagata and Takebe, 1970; Button and Botha, 1975; Potrykus *et al.*, 1977). Plants regenerated from protoplasts have been reported for rapeseed (Katha *et al.*, 1974). Besides regeneration study, the cell wall-less protoplast also gives us a tool for approaching plant physiology, somatic hybridization and plant breeding (Bajer, 1973; Carlson, 1973; Holl, 1975).

Chinese cabbage (*Brassica pekinensis* Rupr.) and Chinese chive (Allium ordorum L.) are important and delicious vegetables. Easy cultivation and rapid growth are the character of Chinese cabbage. Emzymatic isolation of Chinese cabbage protoplast has been reported (Tseng *et al.*, 1975). But the isolation of protoplasts from Chinese chive has not been previously achieved. In the present study, we have tried to isolate protoplasts of Chinese chive and at the same time to observe the fusion of protoplasts from both plants.

MATERIALS AND METHODS

A. Plant materials

Both Chinese cabbage and Chinese chive were bought on the local market and kept in a refrigerator at 5-10°C. Taking off the outer and older leaves of the head, the innermost young leaves of Chinese cabbage were immersed in 70% ethanol for 1 minute, then washed three times with sterilized double-distilled water, then soaked in 2% sodium hypochlorite for 20 minutes, and finally washed three times. Samples of Chinese chive were prepared in the same manner except that specimens were collected from young leaves and the whitish-yellow region of the young pedicels.

B. Protoplast isolation

The enzymatic isolation of protoplasts followed the method of Power and Cocking (1970). One to two grams of sterilized leaves were cut longitudinally into small pieces. The leaf segments were put into a 50 ml Erlenmeyer flask containing 10 ml of enzyme mixture (Table 1), and the flasks were incubated in a rotary shaker (at 25 rpm) at room temperature. At different time intervals small amounts of the sample were removed with a pipette to check protoplast separation and these were counted with a haemacytometer. After 4 hours of in-

cubation, the protoplasts were separated by filtration through a 4-layered nylon cloth. The isolated protoplasts in 13% mannitol solution were centrifuged at speed-mark 5 of EBA III centrifuge for 5 minutes. Sedimented protoplasts were washed twice with 13% mannitol containing 0.07 M $CaCl_2$. The washed protoplasts thus prepared were stored in a refrigerator for further experiment. The isolated protoplasts in 25% sucrose mixture were often seen floating on the surface of the liquid after standing for 30 minutes. The floating protoplasts on top of the solution were collected and washed by adding 3 volumes of 13% mannitol containing 0.07 M $CaCl_2$ and centrifuged at speed 5. Then these protoplasts were again washed in 13% mannitol, centrifuged and stored in a refrigerator.

C. Protoplast fusion

a. Fusion of protoplasts on a depressed slide

Protoplasts from Chinese cabbage and Chinese chive in equal quantities (ca 150 μl) were pipetted onto a cover glass. After the protoplasts had settled on the cover slip to form a thin layer, 100 μl of 56% polyethylene glycol (PEG, m. w. 4000, pH 10.5) was slowly added, then incubated at room temperature for 50 minutes. After incubation the cover slip containing mixtures was put on a depressed slide and observed under low and high power. The frequency of spontaneous and induced fusion of the protoplasts was compared. For studying heterokaryotic protoplast fusion, one of the two types was prestained with neutral red (0.05%). The frequency of homokaryotic and heterokaryotic fusions was then recorded.

b. Fushion of protoplasts in a test tube

To one ml of protoplasts (10^6/ml) in solution in a test tube added 0.5 ml of 56% PEG solution (pH 10.5). The mixture was gently shaken for 5 seconds and then permitted to stand for 10 minutes, this was then centrifuged by EBA III at speed 3 for 3 minutes. The pellets were collected and washed with MS medium (Murashige and Skoog, 1962), and a drop of protoplast suspension was placed on a slide and observed under the microscope.

Table 1. Enzyme solutions for protoplast isolation.

Enzymes	Osmotics	pH
(a) 4% Cellulase 2% Pectinase	13% Mannitol	5.8
(b) 4% Cellulase 2% Pectinase	25% Sucrose	5.8

RESULTS

A. Isolation of protoplasts

Protoplasts isolated from various tissues of different plants show variation in size and quantity (Figs. 1A, 1B, 1C, 3A and 3B). In the first hour after incubation in an enzyme mixture, plasmolyzed cells are usually still found in tissue segments. Some of these are separated into groups of cells (Figs. 2A and 2B). Individual protoplasts without a cell wall are also found. The number of protoplasts increases as the incubation period is increased (Figs. 1A, 1B and 1C). After 4 hours of incubation a maximal number of protoplasts can be collected from various tissues. The average yield of protoplasts is about 10^9 per gram of fresh leaf segments in Chinese cabbage, but in Chinese chive is about 10^6 protoplasts per gram material. The quality of osmoticum also affects the amount of living protoplasts collected. In 13% mannitol mixture the yield of protoplasts is much higher than that in 25% sucrose solution. In sucrose osmoticum, dead or fractured protoplasts are more often observed and more protoplasts are lost during isolation.

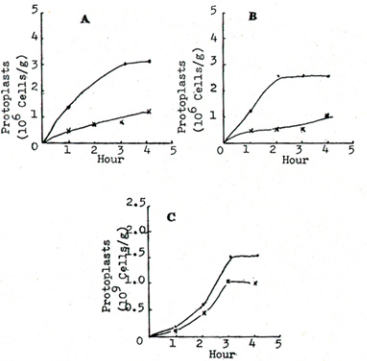

Fig. 1. Protoplasts isolated from different tissues of plants in different osmotica. (—•—• denotes 13% Mannitol and —×—× denotes 25% Sucrose)
A. Pedicel of Chinese chive B. Young leaves of Chinese chive
C. Young leaves of Chinese cabbage

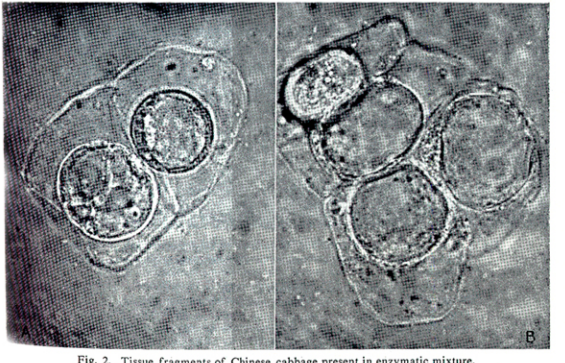

Fig. 2. Tissue fragments of Chinese cabbage present in enzymatic mixture.
A. 2-celled fragment (×1030) B. 4-celled fragment (×1030)

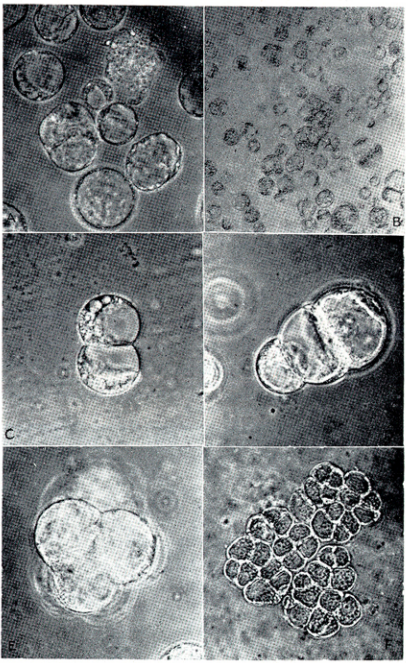

Fig. 3. Intraspecific fusion occurring in isolated protoplasts (A and B are spontaneous fusion. C to F are fusions of Chinese cabbage protoplasts induced by PEG.)

A. Protoplasts of Chinese cabbage (×1030)
B. Protoplasts of Chinese chive (×260)
C. 2 aggregating protoplasts (×1030)
D. 3 aggregating protoplasts (×1030)
E. 8 aggregating protoplasts (×1030)
F. Aggregating groups consisting of 3 and 4 protoplasts (×260)

As shown in Figs. 3A and 3B, living protoplasts of Chinese cabbage and Chinese chive are spherical in shape and their sizes range from 3 to 10 μm and 2.5 to 8 μm, respectively. Some protoplasts with prominent plastids were observed. Most of them contained a large and permanent vacuole and the cytoplasm was squeezed to the peripheral region. Cytoplasmic streaming is ordinarily seen in the peripheral cytoplasm, but sometimes a branching type of streaming occurs between the vacuoles. Isolated protoplasts can be well preserved in osmoticum at 5–10°C for a long period.

B. Protoplast fusion

The protoplasts ready for fusion may be of the same size (Fig. 3C), but protoplasts of different sizes were also observed (Fig. 3D). In general, two protoplasts are seen fusing but

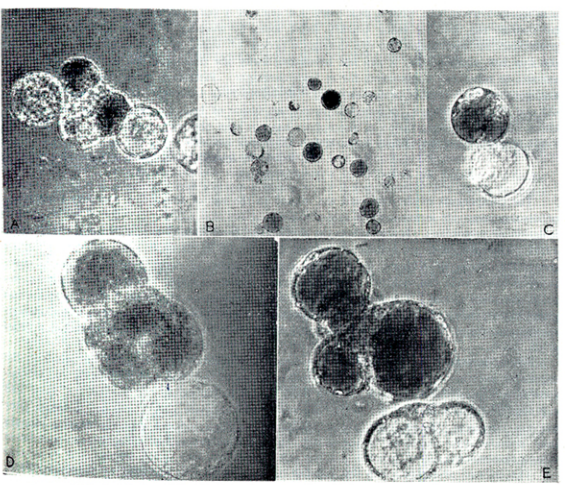

Fig. 4. Vital staining for detecting protoplast fusion.
A. Homokaryotic fusion of Chinese cabbage protoplasts (×1030)
B. Prestained protoplasts of Chinese cabbage and unstained protoplasts of Chinese chive before PEG treatment (×1030)
C. Contacting protoplasts of two species before PEG treatment (×1030)
D. 5 aggregating protoplasts of Chinese cabbage and one contacting protoplast of Chinese chive (×1030)
E. 4 aggregating protoplasts of Chinese cabbage and 2 fusing homokaryotic protoplasts of Chinese chive (×1030)

sometimes three or even more than three are seen in a fusion group (Figs. 3E and 3F). The frequency of spontaneous fusion is very low (4%) within 10 minutes after isolation, but it increases as the incubation time was increased. In Chinese cabbage, the frequency of spontaneous fusion increased up to 30% (210/747) after 48 hours of incubation.

A high molecular weight of PEG (m. w. 4000) is very handy for the induction of fusion between protoplasts. PEG not only increases the frequency of aggregation up to 50~70% (depending on protoplasts density), but also increases the number of protoplasts in aggregating group (Fig. 4). Almost all of them are two-celled fusions, but three, four or five protoplasts are also frequently observed in fusion groups (Figs. 3D and 3F). In one extreme case, eight protoplasts were present in one group (Fig. 3E). The duration of PEG treatment shows the successive steps of protoplast fusion. Vital staining of protoplasts with 0.05% neutral red can distinguish a living cell from a dead one. Contents of a dead cell can be stained immediately by neutral red, but its color quickly fades away by washing. On the contrary, the living protoplasts can accumulate the dye through a period of time, up to 30 minutes. But once the dye has accumulated, it can not be washed away and protoplast can keep the dye in the vacoule or other cell components for at least overnight. Prestained protoplasts can easily be distinguished from regular protoplasts (Fig. 4B) before PEG treatment. As shown in Figs. 4A, 4D and 4E, PEG induces intraspecific protoplast fusion of both Chinese cabbage and Chinese chive. But intergeneric fusion of protoplasts has not been observed, even though two different kinds of protoplasts may be in close contact (Figs. 4D and 4E).

DISCUSSION

The components of the cell wall are related to the age of the leaves and vary from stage of development. It seems to be a critical threshold for the success or failure in enzymatic isolation of protoplasts. It also affects the duration of protoplast isolation (Nagata and Takebe, 1971). The mosophyll protoplasts of Chinese cabbage are much more easily released from the tissues than those of Chinese chive. The average number of protoplasts from Chinese chive is much lower than the yield of protoplasts from Chinese cabbage. This may possibly be due to its succulent tissues. The direct method of treating tissues with cellulase and pectinase, has given higher yields of protoplasts than the two-step method, but the protoplasts often undergo spontaneous fusion with protoplasts of an adjoining cell by means of the plasmodesmata (Kao *et al.*, 1974).

The concentration of enzymes and the duration of incubation period are important in the successful isolation of protoplasts. High concentrations of enzymes and prolonging the incubation of protoplasts in an enzyme mixture often results in increasing the number of dead and ruptured protoplasts (Kao *et al.*, 1974). Ruptured cells elutriate a lot of the cell contents after enzyme treatment (Nagata and Takebe, 1970). These facts indicate that the enzyme mixture may contain some unknown factor which changes the properties of the plasma membrane during incubation.

The isolated protoplasts can be induced to fuse with one another by employing PEG, which often is used as an adsorbent in the column of gas chromatography. PEG, m. w. ranging from 1500 to 6000, may act as a molecular bridge between the surface of the adjacent protoplasts and increase the frequency of protoplast aggregation, which is the initial step in membrane fusion (Withers and Cocking, 1972). Both intra and interspecific fusion can be enhanced by PEG. Higher concentration of PEG will increases the degree of protoplast fusion, but it also increases the number of ruptured cells. The optimal concentration of PEG for protoplast fusion is between 15% and 25%. By prolonging the time the protoplasts are incubated in and emzyme solution, the fusion frequency increases because their capacity for cell wall regeneration is reduced.

The bivalent calcium ion is very important in the preservation of protoplasts during the processes of isolation and purification. The calcium ion has been suspected of inhibiting the activity of pectinase resulting in the failure of isolating protoplasts from Chinese chive (Tseng *et al.*, 1975). In our studies, we found that a high concentration of calcium ion (0.08 M) in the isolation medium does not affect the isolation of protoplasts from Chinese chive, Chinese cabbage or rice. In the presence of the calcium ion, the protoplasts can tolerate a stronger centrifugal force during purification. Fusion solutions containing calcium salts lower the fusion rate regardless of whether an enzyme solution is present or not (Kameya and Takahashi, 1972). But in a combination treatment with PEG and calcium ions, the fusion frequency is enhanced by the addition of a calcium salt. This phenomenon is in agreement with the statement of Bajaj (1977).

Protoplast fusion between different animals (Harris, 1970), different plants (Bajaj, 1977) and between animal and plant protoplasts (Ahkong *et al.*, 1975) after fusogen treatment has been observed. However, in our experiments, though protoplast fusion from different tissues of same species often occurs, no intergeneric fusion of protoplasts was observed.

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