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The study on the isolation and culture of the protoplast from heterothallic *Closterium*

Takuya NAKAYAMA, Eriko ISHII and Hajime KANAZAWA

The enzymatic isolation of protoplasts and their culture were studied in heterothallic strains of *Closterium peracerosum-strigosum-littorale* complex. The digestion of their cell walls proceeded from division scars distributed along the cell wall, and protoplasts were released out from one of these scars with remaining portions of the cell wall undigested. The yield of the protoplast varied according to cell age. When "postculture" (stationary culture with fresh medium overnight) was carried out prior to enzyme treatment, protoplasts were obtained in high yield from the cells of various ages. The maximum yields of the protoplast of mt^+- and mt^--cells were 80-90% and 70-80%, respectively, in this study. The cultivation of protoplasts in the presence of 100 mM $CaCl_2$ led them to initiate the regeneration of the cell walls. Scanning electron microscopy revealed that the early stages of the cell wall regeneration in this alga was similar to that in higher plants. Several physiological phenomena concerning the culture of the protoplasts of this alga were also examined.

Protoplasts have been isolated enzymatically from many kinds of plants, cultured and regenerated into new individuals. Then they have been used routinely as an important experimental tool in the understanding of physiology, development and morphogenesis of plant cells. Most of these studies, however, have been done with higher plants, and protoplasts of lower plants have received less attention.

Recently protoplasts have been isolated from many eukaryotic algae (reviewed by BERLINER, 1981, 1983). With regard to desmids, protoplasts have been derived from *Cosmarium* (CHARDARD, 1972), *Micrasterias* (BERLINER and WENC, 1976), *Staurastrum* and *Netrium* (BERLINER, 1978). For the *Closterium* species, protoplasts have been isolated in *C. acerosum* (BERLINER, 1978) and *C. peracerosum-strigosum-littorale* complex (KATO *et al.*, 1983). All these reports described mainly protoplast obtentions, but dealt little with cultures and regenerations of protoplast.

Closterium species are interesting materials to study some biological problems. Several workers have studied morphologically, physiologically and genetically about several problems using *Closterium*. Use of protoplast may contribute to these studies. For example, the protoplast may be used to

observe the developmental process of cell wall, to enable organelle to be isolated easily, and to make artificial zygote by cell fusion.

In this paper, an improved preparation of protoplast in higher yield than those of previous reports (BERLINER, 1978; KATO *et al.*, 1983) is shown. Some physiological natures of the protoplasts including the ability to regenerate the cell wall will also be discussed.

Materials and Methods

Algal materials and culture conditions

The *Closterium peracerosum-strigosum-littorale* complex, strain KAS-4-29 (mating type minus clone, mt^-) and KAS-4-30 (mating type plus clone, mt^+), were used. Each of the clone was cultured in 50 ml of modified Ichimura's C-medium (1971) in a 100 ml-Erlenmeyer flask with rotary shaking (100 rpm) under a regime of 16 h of light (ca. 3,000–4,000 lux with fluorescent tubes, 25° C) and 8 h of darkness (20° C) (KATO *et al.*, 1981). Cells were subcultured every fifteen days. About 1×10^5 cells in 1 ml of the cell suspension for each clone were inoculated into the flask and the growth culture was initiated.

"Postculture" of vegetative cells

Prior to the preparation of protoplast, cells were pretreated as follows if necessary. After harvesting the cells by centrifugation ($500\times g$, 1 min), they were resuspended in the fresh C-medium and were recultured without shaking at low light intensity (ca. 700–900 lux) overnight prior to protoplast induction while the other conditions were the same as described above. Hereafter it will be called as "postculture".

Preparation of protoplast

Cells were harvested at the beginning of the light period of the aimed growth stages by centrifugation ($500\times g$, 1 min) and washed twice with water. The cells were then resuspended in water again.

The standard enzyme solution consisted of 2% (w/v) cellulase ONOZUKA R-10 (Yakult Pharmaceutical Industry Co., Ltd.), 1% (w/v) macerozyme R-10 (Yakult Pharmaceutical Industry Co., Ltd.), 0.6 M sorbitol and 0.06 M MES in water; pH was adjusted with NaOH to 5.5. The enzyme solution was sterilized by filtration (pore size, 0.22 μm) when the culture of the protoplasts was carried out.

Equal volumes of the cell suspension and the enzyme solution were mixed together and incubated without agitaion at 25° C for 3 h (standard incubation period). Yields of protoplast were determined by the percentage of protoplasts in the total number of counted cells.

Culture of protoplast

After enzyme treatment for 3 h, protoplasts were harvested by centrifugation ($50 \times g$, 1.5 min), washed twice with protoplast culture medium, that is, the C-medium containing 0.1 M $CaCl_2$ and 0.3 M sorbitol, and resuspended in the same medium. The cells were cultured in 4 well multidishes (NUNC No. 134673) stood stationary under a regime of 16 h of light (25° C) at various light intensity (darkness, ca. 700 lux, ca. 3,000 lux) and 8 h of darkness (20° C). The cell density was adjusted to 5.2×10^4 cells in the 400 μl medium per a well.

Detection of cell wall and determination of viability of protoplast

Calcofluor White M2R (Polysciences, Inc., Warrington) dissolved in the protoplast culture medium was used to stain the cell wall to confirm that isolated protoplasts had no cell wall. This dye was also used to follow the process of cell wall regeneration of protoplast.

To examine protoplast viability, the protoplasts were observed with an inverted microscope during the protoplast culture. The living protoplasts could be easily distingushed from the died one, because the living cell was round in shape, had smooth membrane, had a bright green color, and had a uniform cytoplasm, although the died one lost its round shape and bright green color, had small droplets or vesicles associated with the wrinkled membrane, and had a granulated cytoplasm. The viability determined by the observation of these points consisted with the results from the observation using fluorescein diacetate (Sigma chemical Co.) or phenosafranine (Wako Pure Chemical Industries, Ltd.) according to WIDHOLM (1972).

Electron microscopic observation

Protoplasts attached on a cover glass coated with poly-L-lysine were fixed in 1% (w/v) glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.4) containing 0.4 M sorbitol overnight or longer at 4° C. After rinse in buffer solution they were postfixed for 1 h in 1% (w/v) osmium tetraoxide prepared in 0.1 M cacodylate buffer (pH 7.4) at 4° C, rinsed with water, and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, 100%). Samples were then dried at critical point from carbon dioxide. The cover glass with dried protoplasts was mounted on a stub and coated with gold using an ion sputter JEOL JFC-1100. Specimens were examined in a JEOL JSM-T20 scanning electron microscope with an accelerating voltage of 20 KV for low magnification and in a JEOL JSM-U3 scanning electron microscope with an accelerating voltage of 25 KV for high magnification.

Results

The process of protoplast formation

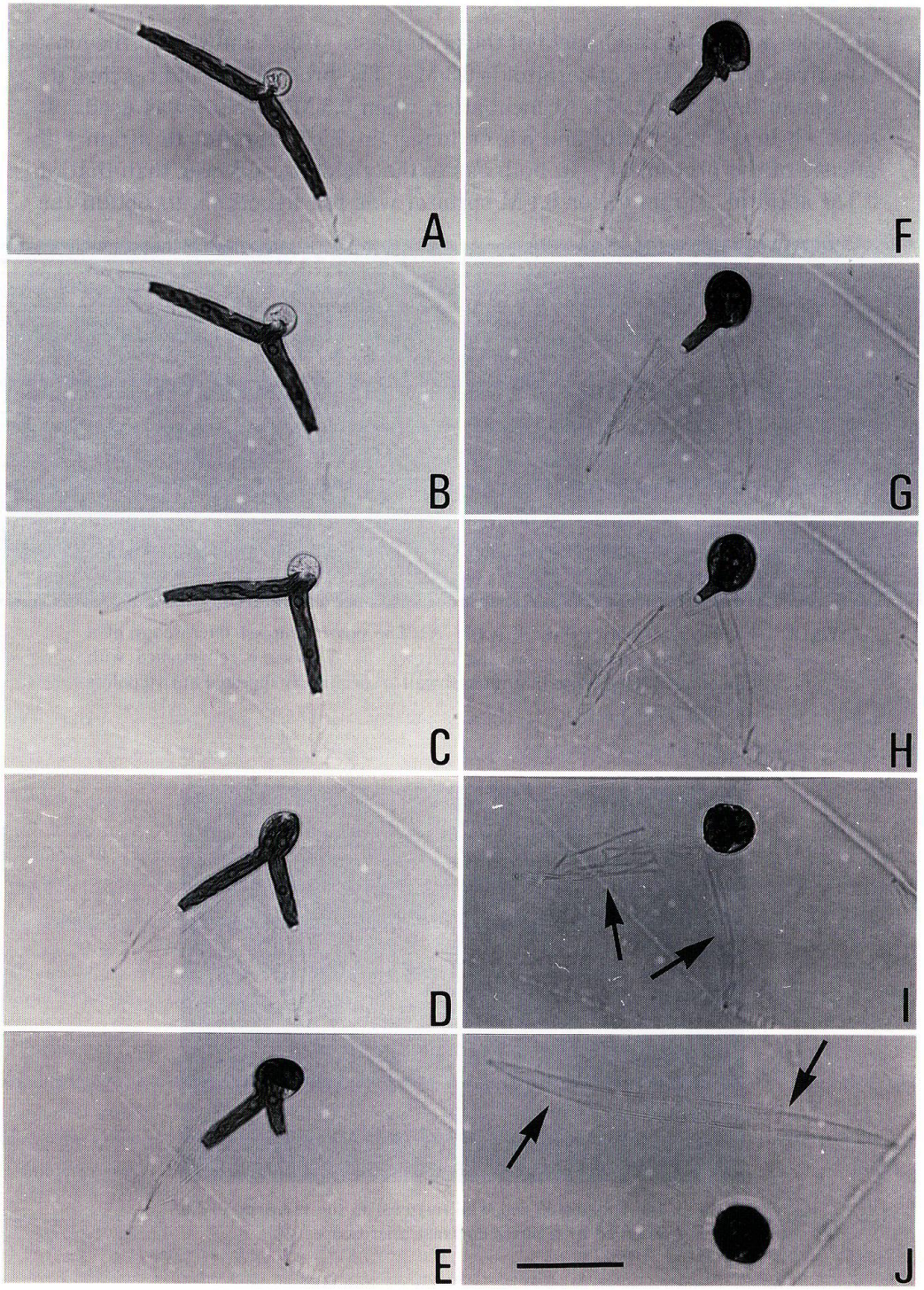
Fig. 1 shows the typical process of protoplast formation. It seems the protoplast comes out from the specifically digested part of the cell wall. First, it was observed the cell membrane swelled out of the digested position slightly distant from the nucleus (Figs. 1A, 2A, arrow). This position seems to be the same place as that where the conjugation papilla (ICHIMURA, 1971) is formed during the mating process. As can be seen in Fig. 2B (arrow 1), the cell wall at this position is specifically digested and the swelling prove to be of the cell membrane because this position dosen't fluoresce by Calcofluor White. In addition to this place, another part of the cell wall is also digested specifically (Fig. 2B, arrow 2). All these specifically digested parts of the cell wall correspond to the division scars which are the sutures between new and old semicell walls occurred by cell division. Next, the swelling increased in size and each semicell protoplasm contracted into the swelling (Fig. 1B -E). At last, the cell wall was broken and divided into two or more pieces, and the protoplast was released (Fig. 1F-J). Just after releasing the protoplast, the cell wall avoiding complete digestion was remained (Fig. 1 I, J, arrows).

It is known the case that 0.1-10 mM calcium ion was added to the enzyme solution to protect the protoplast membrane against any injuries during protoplast isolation (OHIWA, 1977 ; KAMEYA *et al.*, 1981 ; BATES *et al.*, 1983 ; VERHOEK-KOHLER *et al.*, 1983). Therefore we tested the effects of calcium ion on protoplast isolation. When 3 mM Ca^{2+} was present in the mixture of cells and enzyme solution, protoplast formation was completely inhibited, while the formation was not inhibited by 30 mM K^{+} and was inhibited slightly by 30 mM Mg^{2+} (Table 1). Fig. 3 shows that the cell wall is preserved completely from the enzymatic digestion in the presence of calcium ion and protoplasm contracts as a result of plasmolysis.

Effects of sugar concentration in enzyme solution on protoplast yield

For the optimization of conditions for protoplast preparation, it was found that the concentration of sorbitol used as an osmotic stabilizer was one

Fig. 1. The process of protoplast formation. A-I : A course of protoplast formation. A, the conjugation papilla-like swelling formation ; B and C, the swelling increased in size ; D-H, the contracting protoplasm and the separating cell wall ; I, a protoplast just released and remaining cell wall without the complete digestion (arrows). J : Another example of protoplast release without the complete digestion of the cell wall (arrows). The bar represents 50 μm.


A
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of the keys to obtain high yield of the protoplast. Fig. 4 shows that the most effective concentration of sorbitol is 0.3 M. The protoplast yield reached its maximum level within 3 h of incubation when 0.3 M sorbitol was used. It took 4 h in 0.4 M sorbitol and 5 h or longer in 0.5 M sorbitol to attain full release of the protoplast. In both cases, the yields were lower than that of 0.3 M sorbitol. Final 0.2 or 0.1 M sorbitol was too hypotonic to obtain the

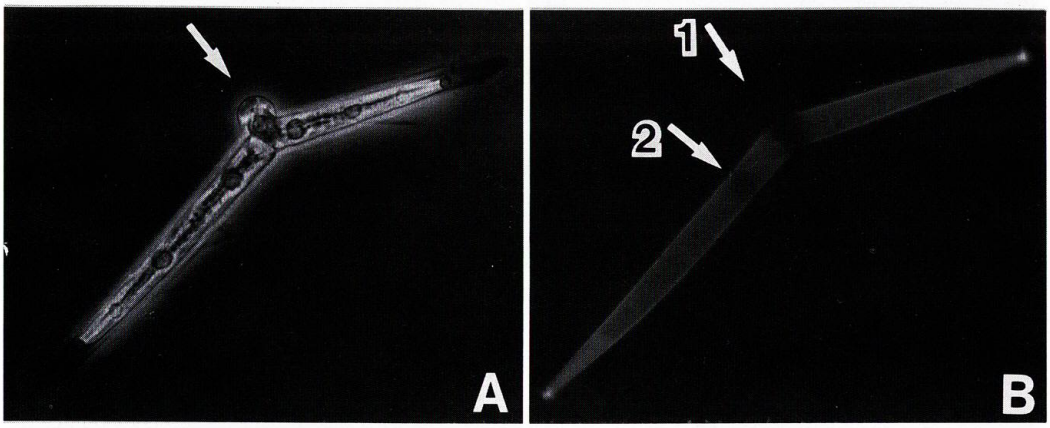

Fig. 2. Partial digestion of the cell wall. A: The phase contrast microscopy of a conjugation papilla-like swelling (arrow). B: The same cell stained with Calcofluor White; note that the cell wall is specifically digested at two points (arrows 1, 2).

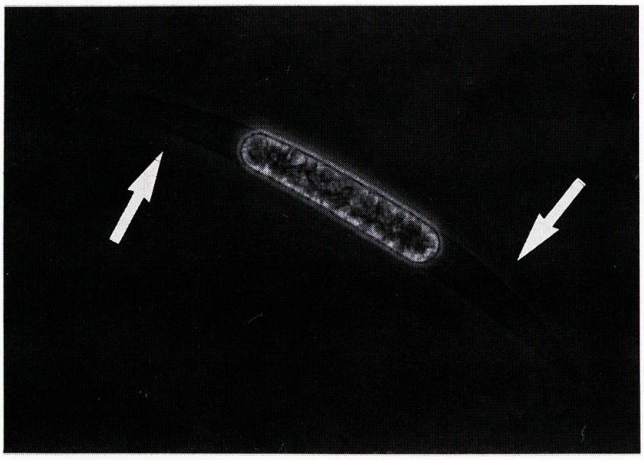

Fig. 3. The undigested cell wall (arrows) in the presence of Ca^{2+} observed by a phase contrast microscope.

protoplast.

The combination of 1% (w/v) cellulase and 0.5% (w/v) macerozyme was appropriate for protoplast formation.

Under the optimum conditions including the best cell culture condition as described below for protoplast preparation, the yield of protoplast reached its maximum level, 80-90% in mt^+- and 70-80% in mt^--cells, within 3 h of incubation (Fig. 4). In general, the yield of protoplast was higher in mt^+- than mt^--cells (Figs. 4, 5, Table 1).

Table 1 Effects of cations on protoplast yields

salts	Yields of Protoplast (%)	
	mt^+	mt^-
3 mM $CaCl_2 \cdot 2H_2O$	2.2±1.2	3.9±0.6
30 mM $CaCl_2 \cdot 2H_2O$	0.3±0.2	0.2±0.2
30 mM $Ca(NO_3)_2 \cdot 4H_2O$	0.3±0.4	0.9±0.5
30 mM KCl	86.4±1.6	79.1±3.9
30 mM $MgCl_2 \cdot 6H_2O$	62.7±4.5	46.2±9.3
control	88.2±0.9	72.7±2.6

Cells harvested after 6 days of growth culture were "postcultured" overnight and treated for 3 h with the standard enzyme solution containing each salt shown above. The results given are the averages ± standard deviations from three independent experiments.

Effects of the growth culture condition on protoplast yield

Protoplasts were derived more effectively from "postcultured" cells than from cells without "postculture". The yield of protoplast became higher by a stationary culture only prior to enzyme treatment without refreshing the medium than by a continuous rotary shaking. And the yield of protoplast reached the maximum level when cells were "postcultured" overnight in the fresh C-medium without shaking (Fig. 5).

The yield of protoplast was depended on age of the cells. As shown in Fig. 5, protoplasts were obtained most effectively about 5 or 6 days after the start of culture, corresponding to middle-late log phase of growth, whether "postcultured" or not. Without "postculture", the yield increased until the peak of the 5th and 6th day of culture for mt^-- and mt^+-cells, respectively, and then decreased gradually with aging. With "postculture", however, protoplasts could be isolated in continuously high yields between the 5th and 8th day of culture in mt^--cells and between the 6th and 9th day of culture in mt^+-

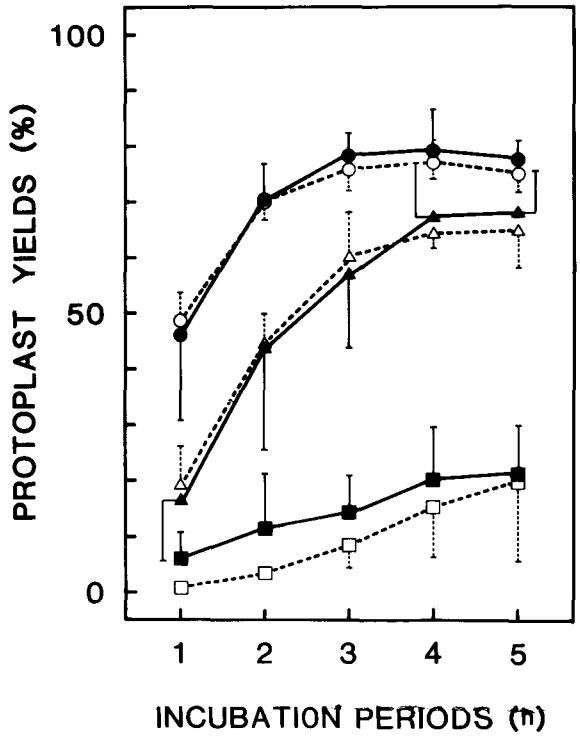

Fig. 4. Effects of sorbitol concentrations of enzyme solution on protoplast yields. Cells were treated with the enzyme solution containing final 0.3 M, 0.4 M, and 0.5 M sorbitol, respectively after 6 days of growth culture and following overnight "postculture". Each one hour until 5 h of incubation, aliquots of cells were sampled and the yields were measured. —●—, mt^+-cells of 0.3 M sorbitol; ---○---, mt^--cells of 0.3 M sorbitol; —▲—, mt^+-cells of 0.4 M sorbitol; ---△---, mt^--cells of 0.4 M sorbitol; —■—, mt^+-cells of 0.5 M sorbitol; ---□---, mt^--cells of 0.5 M sorbitol. Vertical bars indicate ± S. D. (n = 4).

cells. The yields were always higher with "postculture" than without it. Thus, the "postculture" was effective for the high yield of protoplast. The effect was more remarkable in mt^--cells than in mt^+-cells (Fig. 5).

Viability of cultured protoplast

Presence of calcium ion in the medium was essential to keep the protoplast alive. As seen in Fig. 6, when the protoplasts were cultured in the C-medium containing 0.1 M $CaCl_2$ and 0.3 M sorbitol, more than 80% of protoplasts were alive on until 6th day of culture whereas $Ca(NO_3)_2$ used instead

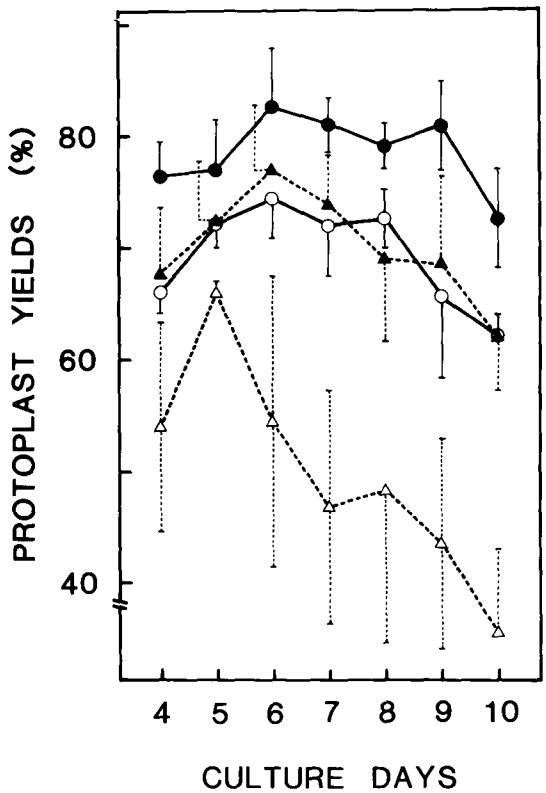

Fig. 5. Effects of "postculture" and cell age on protoplast yields. Cells were treated with the standard enzyme solution for 3 h. In the "postcultured" cell, by way of example, 6 days of culture means 5 days of growth culture and the following 1 day (overnight) of "postculture". —●—, "postcultured" mt^+-cells; —○—, "postcultured" mt^--cells; ---▲---, "non-postcultured" mt^+-cells; ---△---, "non-postcultured" mt^--cells. Vertical bars indicate ± S. D. (n=3-6).

of $CaCl_2$ slightly reduced the viability (Fig. 7A). When $CaCl_2$ was replaced with $MgCl_2$, about 70-80% of protoplasts died within 2 days of culture (Fig. 7B). Almost all cells died within 2 days of culture in the C-medium containing only 0.55 M sorbitol which is isotonic to 0.1 M $CaCl_2$ + 0.3 M sorbitol (Fig. 7C). These results suggest that the calcium ion plays important role(s) in culture of protoplasts. When glucose was used instead of sorbitol, the protoplasts died within 4-8 days of culture in spite of the presence of calcium ion. No effect of $1\text{-}5\times10^{-6}$ M 2, 4-D in the culture medium was seen on survival of protoplasts.

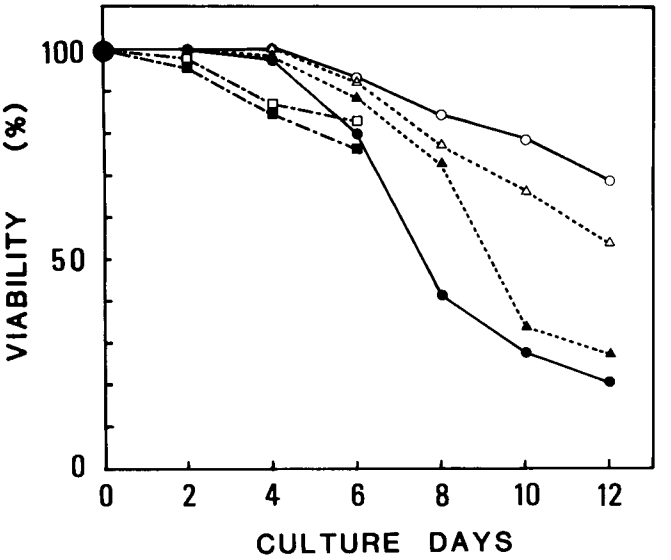

Fig. 6. Viability of cultured protoplasts. Protoplasts were prepared and cultured as described in Materials and Methods after 6 days of growth culture and following overnight "postculture". These results given are the averages from duplicated determinations (n=8-14). —●—, mt^+-cells cultured at 3,000 lux; —○—, mt^--cells cultured at 3,000 lux; ---▲---, mt^+-cells cultured at 700 lux; ---△---, mt^--cells cultured at 700 lux; -·-■-·-, mt^+-cells cultured at darkness; -··-□-··-, mt^--cells cultured at darkness.

Some physiological differences could be seen between mt^+- and mt^--protoplasts. In general, mt^--protoplasts were more viable than mt^+-protoplasts (Figs. 6, 7A). After 6 days of culture, the number of living protoplasts decreased rapidly, especially in mt^+-cells and the dependency on light intensity was different between both mating types of cell (Fig. 6); mt^--cells were more viable at 3,000 lux than at 700 lux, although mt^+-cells were less viable at 3,000 lux than at 700 lux. In darkness, the viability began to be reduced gradually just after the start of culture in both mating types of cell (Fig. 6).

Regeneration of Calcofluor-detectable cell wall

In preliminary experiments, the rate of cell wall regeneration detected with Calcofluor was very low; 0.4-8%. To get higher rate of cell wall regeneration, we obtained the recloned and selected mt^+- and mt^--cells. Their protoplasts could regenerate the cell wall at the rate of more than 70% in mt^+-cells and 25% in mt^--cells (Fig. 8A, B).

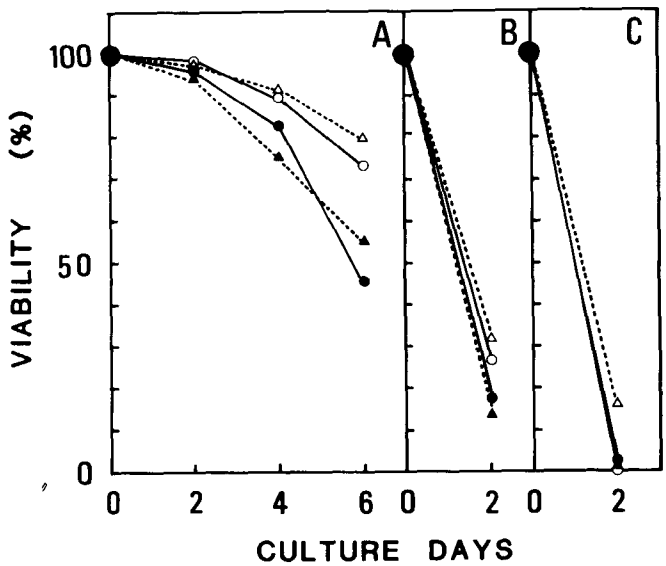

Fig. 7. Viability of protoplasts cultured in the modified medium. The conditions of preparation and culture of protoplasts are the same in Fig. 6. A: 0.1 M $Ca(NO_3)_2$+0.3 M sorbitol. B: 0.1 M $MgCl_2$+0.3 M sorbitol. C: 0.55 M sorbitol. —●—, mt^+-cells at 3,000 lux; —○—, mt^--cells at 3,000 lux; ---▲---, mt^+-cells at 700 lux; ---△---, mt^--cells at 700 lux. (n=8-20).

As shown in Fig. 8A, in mt^+-cells, about 70% of protoplasts had faint Calcofluor fluorescences on whole cell surfaces which were distinguished from the bright red autofluorescences of chloroplasts of naked protoplasts in blue light within 2 days of culture. Until 4th day of culture, about 75% of protoplasts began to regenerate their cell walls and many of them had clear blue-white Calcofluor fluorescences, suggesting the regenerations were well developed. Sometimes the cells which had Calcofluor fluorescences on only hemisphere surfaces could be observed. At 6th day of culture, the rate of bright fluoresced cells were higher in comparison with that of the cells cultured for 4 days. On the other hand, the number of cells which projected the cytoplasm and bursted to die increased up to 6 days of culture. Some of them were observed as budding cells and the cytoplasts were sometimes released from them. The rate of fluoresced cells was almost the same between the cells cultured at 700 and 3,000 lux. In general, however, the protoplasts cultured at 3,000 lux had brighter Calcofluor fluorescences than those cultured at 700 lux. Protoplasts cultured in darkness also regenerated

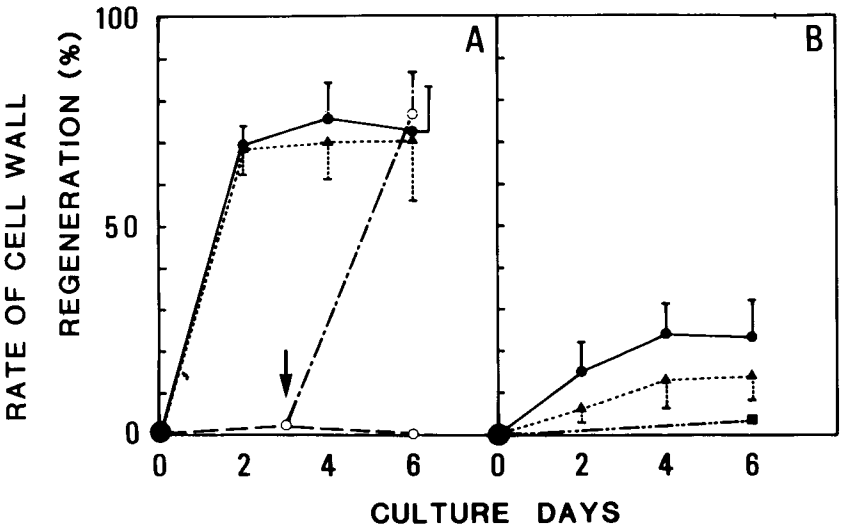

Fig. 8. Rate of cell wann regeneration detected by Calcofluor White. Protoplasts were isolated and cultured as described in Materials and Methods. A: mt^+-cells cultured at 3,000 lux (—●—); at 3,000 lux with 1 mm coumarin - - ○ - -); at 3,000 lux and the medium was exchanged for coumarin-free medium at arrow point (-·-·-); at 700 lux (···▲···). B: mt^--cells cultured at 3,000 lux (—●—); at 700 lux (···▲···); at darkness (-··■··-). Vertical bars indicate ± S. D. (n=3).

the cell wall at the same rate in light, although their cell walls had weak Calcofluor fluorescences even after 6 days of culture.

The cell wall regeneration was inhibited completely by 1 mM coumarin in the protoplast culture medium in mt^+-cells (Fig. 8A). This inhibition was reversible. When the protoplasts were cultured in the medium containing 1 mM coumarin for 3 days at 3,000 lux, no cell could regenerate the cell wall. After another 3 days of culture without coumarin, more than 75% of cells became to have bright blue-white Calcofluor fluorescences. But when the protoplasts were cultured for another 3 days with coumarin, no cell still could regenerate the cell wall.

In mt^--cells, the cell wall regeneration rate at 3,000 lux was higher than at 700 lux (Fig. 8B). And cell wall regeneration was inhibited completely not only in darkness (Fig. 8B) but also by 0.5 mM coumarin (data not shown).

Scanning electron microscopic observation of the regeneration of the cell wall

There was no essential difference in the process of the cell wall regeneration between both mating types of cell. The regeneration didn't proceed synchronously by the methods used here. Different stages of regeneration

were observed even in the same preparation. The cell wall regeneration during 4 days of culture proceeded as follows.

As shown in Fig. 9, freshly isolated protoplast had smooth and uniform surfaces. First, many small projections appeared on the cell surface as described in higher plants, tobacco and grapevine (BURGRESS and LINSTEAD, 1976)(Fig. 10, arrowheads). Next, the fibrillar structures appeared (Fig. 11) and some of which seemed to be associated with small projections (Fig. 11, arrowheads). Then the fibers elongated to be entangled gradually (Figs. 12, 13). And big spherical projections sometimes became to be seen (Fig. 14, arrowheads). At the 4th day of culture, the fibers became to be well densed and made the fibrous mat over whole cell surface (Fig. 15A, B). But in some cases, the fibers concentrated on a certain part of the cell surface and made fibrous mats (Fig. 16, arrowheads) which could be detected with Calcofluor as blue-white fluorescing spots. However, the complete cell wall as seen in vegetative cell couldn't be observed within 4 days of culture.

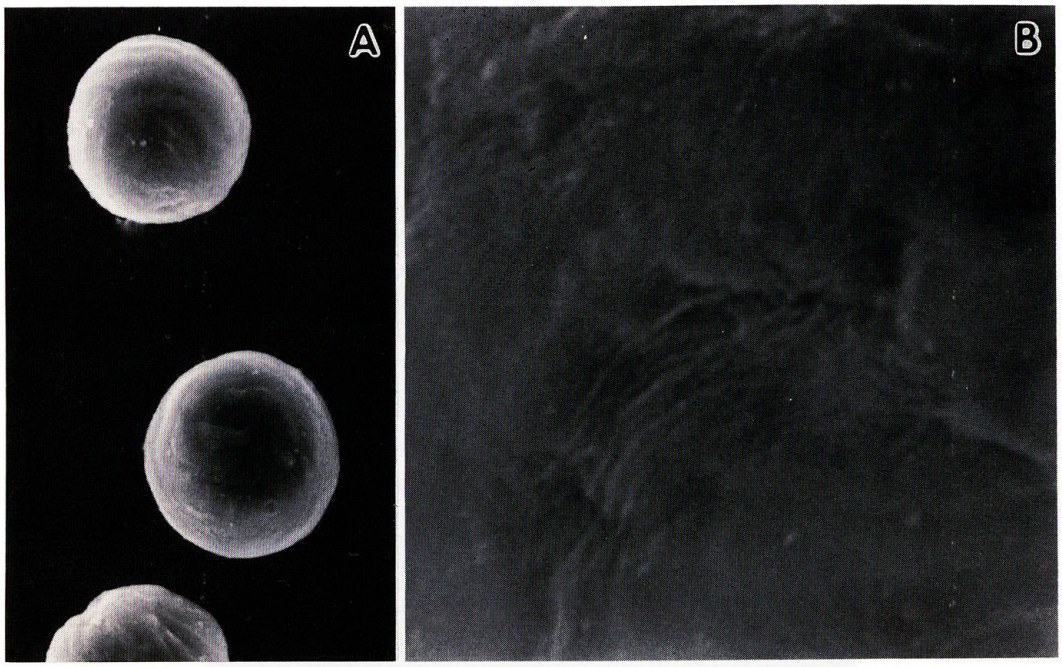

Fig. 9. Scanning electron microscopy of freshly isolated mt^+-protoplasts. A: Whole cells, × 1,300. B: A part of cell surface (the furrows are the artifacts which occurred during the observation), × 20,000.

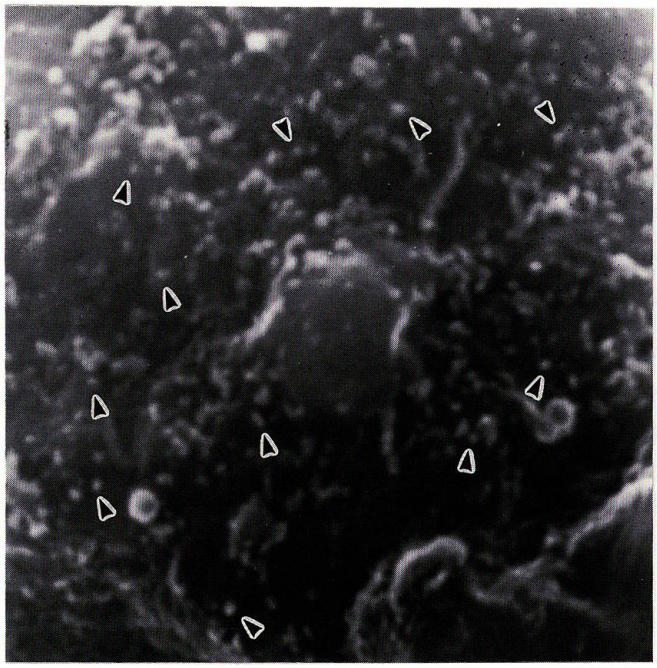

Fig. 10. Scanning electron microscopy of the mt$^+$-protoplast cultured for 2 days. Many small projections are seen on the cell surface (arrowheads), × 20,000.

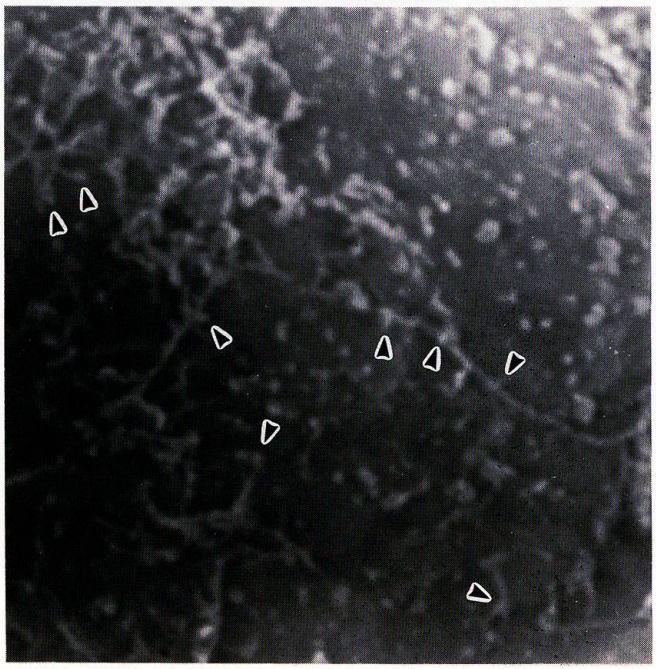

Fig. 11. Scanning electron microscopy of the mt$^+$-protoplast cultured for 2 days. Fibrillar structures, some of which seem to be associated with small projections, are seen (arrowheads). × 20,000.

Fig. 12. Scanning electron microscopy of the mt^+-protoplast cultured for 2 days. The fibers increase in number. × 20,000.

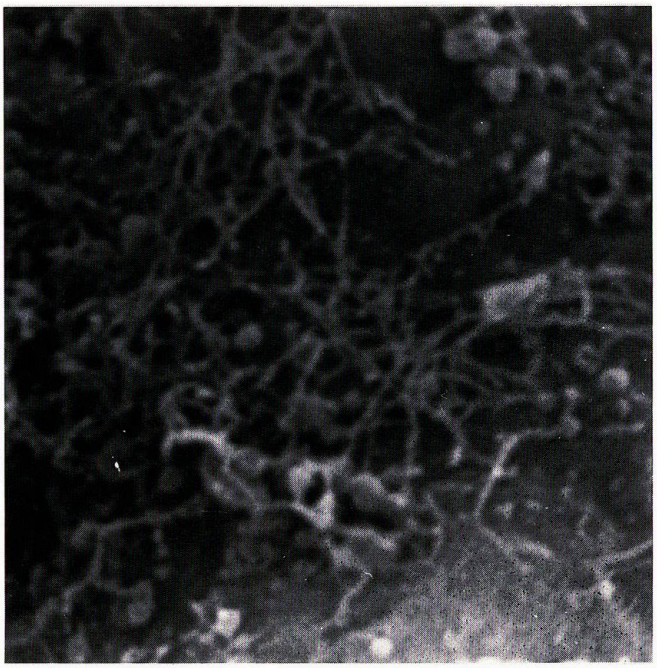

Fig. 13. Scanning electron microscopy of the mt^--protoplast cultured for 4 days. Elongated and entangled fibers are seen. × 20,000.

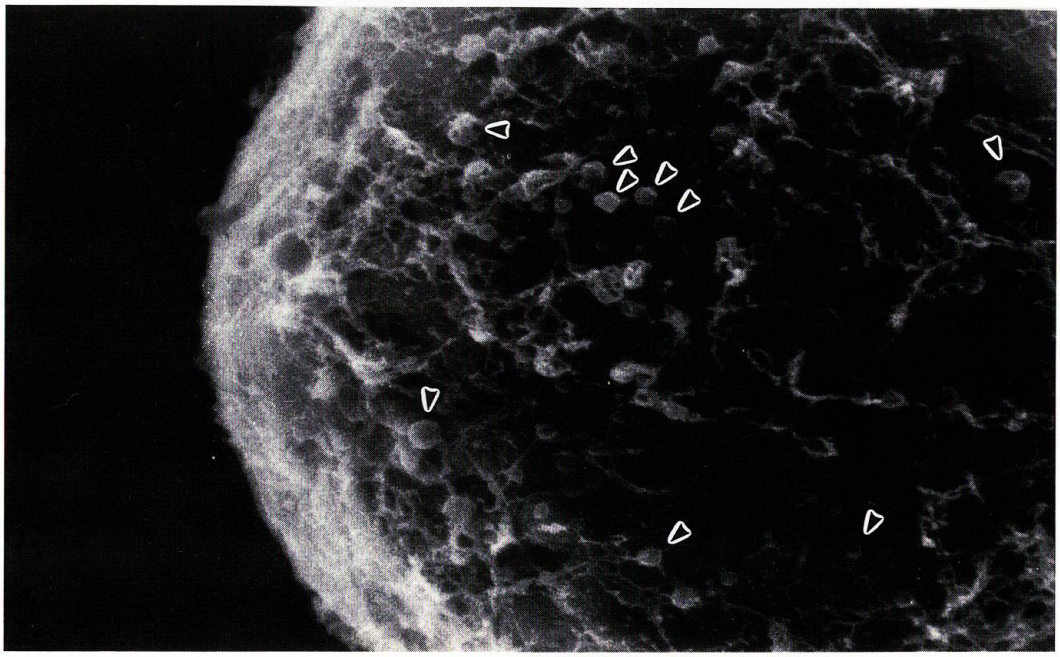

Fig. 14. Scanning electron microscopy of the mt⁻-protoplast cultured for 4 days. Big projections (arrowheads) and well developed fibers are seen. × 10,000.

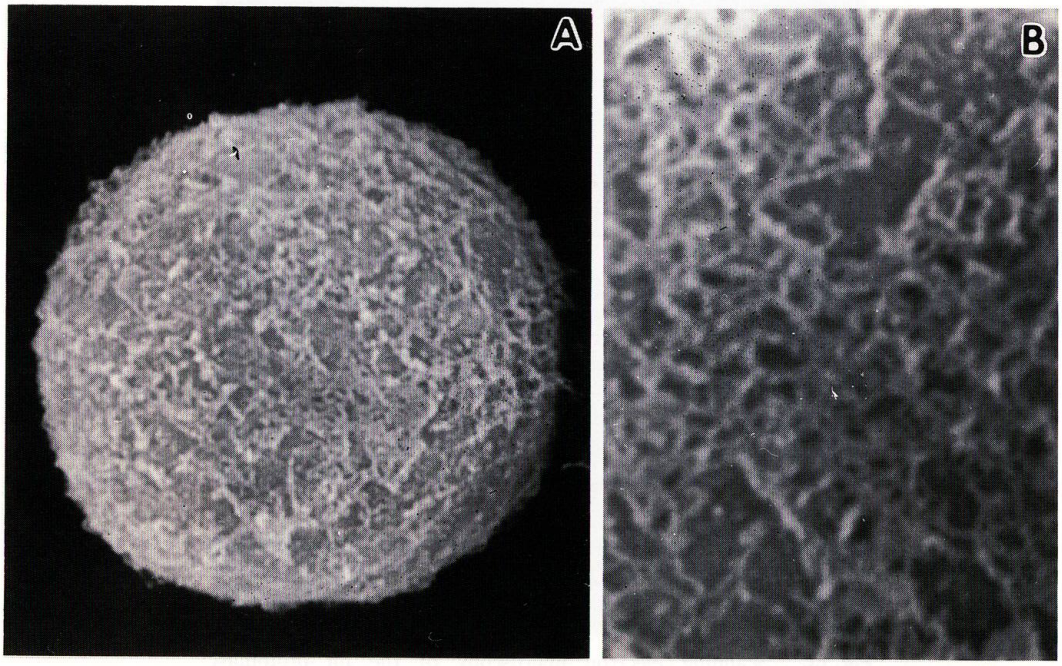

Fig. 15. Scanning electron microscopy of the mt⁻-protoplast cultured for 4 days. Fibrous mat over whole cell surface made by densed fibers are seen. A: Whole cell, × 5,000. B: A part of cell surface, × 20,000.

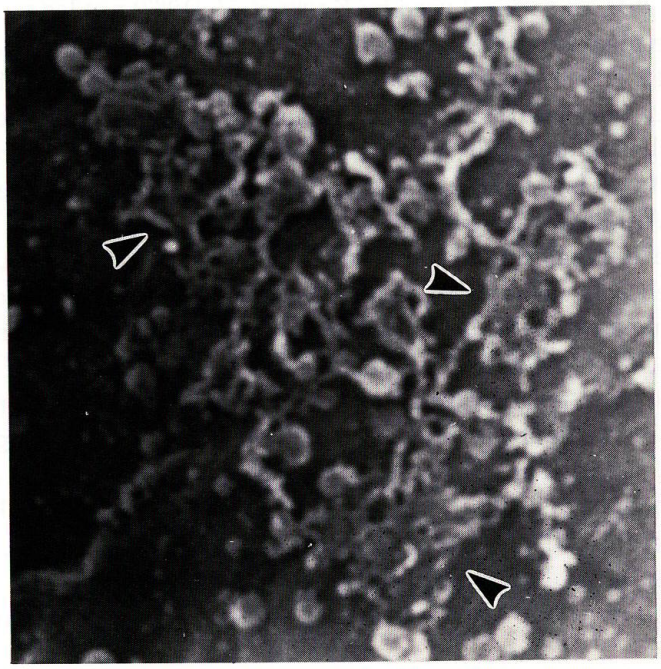

Fig. 16. Scanning electron microscopy of the mt⁻-protoplast cultured for 4 days. The fibrous mats concentrate on one part of the cell surface (arrowheads). × 20,000.

Discussion

Site-specific digestion of the cell wall observed here during enzymatic protoplast isolation is thought to be common nature to *Closterium* species because the site is the division scars which exist on the cell wall of all *Closterium* species. Only one protoplast per cell was observed in this species used here, although in some cases two protoplasts emerged from one cell whose cytoplasm was already divided in two while the cell wall was not divided, just before cell division. However, BERLINER (1978) reported that more than one protoplast per cell was frequently released in *C. acerosum*. It is thought that they would emerge out of different digested parts of division scars at the same time, and that only one of them would be nucleated and the others would be cytoplast, not protoplast.

The optimum concentration of sorbitol as an osmotic stabilizer for protoplast preparation is 0.3 M (Fig. 4). Using 0.3 M sorbitol, the yield of protoplast attained its maximum level within 3 h whereas high concentra-

tions, 0.45–0.8 M, of sorbitol used by KATO *et al.* (1983) needed more than 10 h to reach the maximum levels which were lower than that of 0.3 M sorbitol.

BERLINER (1978) reported that the yield of protoplast varied from 30 to 90%. Under the culture conditions including "postculture" used here, the yield is stable and high ; 70–80% in mt^--cells and 80–90% in mt^+-cells. Thus, the "postculture" is effective to get the high yield of protoplast, especially in mt^--cells. Although the reason why "postculture" is effective is not known, the possible explanation is as follows. It was observed that the "post-cultured" cell pellet was more densed than the "non-postcultured" one after twice washing with water by centrifugation before enzyme treatment. This suggests that the mucilage of cell surface is more easily taken off by washing when the cell was "postcultured." Therefore the enzyme solution can act on the cell surface without the interference of mucilage, so producing protoplast with higher yield. The reason why the "postculture" is more effective in mt^--cell is thought to be that mt^--cell usually has more mucilage than mt^+-cell which protect the cell wall from the action of exogenous enzymes, so the yield of protoplast is low without "postculture", but when the cell was "post-cultured", the mucilage is taken off and the protoplast is obtained in high yield.

The presence of calcium ion inhibited protoplast formation completely in *Closterium* (Fig. 3, Table 1). This is thought not to be only the inhibition of the action of the enzymes by calcium ion, because protoplasts from many kinds of plant were obtained enzymatically in the presence of calcium ion (OHIWA, 1977 ; KAMEYA *et al.*, 1981 ; BATES *et al.*, 1983 ; VERHOEK-KOHLER *et al.*, 1983). In *Closterium*, calcium ion may play the unknown role on the composition of cell wall.

Calcium ion played the important role(s), also in the survival of protoplast and the cell wall regeneration. When 0.1 M $Ca(NO_3)_2$ was used as the source of calcium ion, the viability of the protoplast was less than when 0.1 M $CaCl_2$ was used (Fig. 7A). This inhibition may be due to the excessive supply of nitrogen. When the medium containing 0.55 M sorbitol, which was isotonic to 0.1 M $CaCl_2$+0.3 M sorbitol, was used for protoplast culture, all protoplasts bursted to die within 2 days of culture. When the medium containing 0.1 M $MgCl_2$+0.3 M sorbitol was used, almost all protoplast died within 2 days although the cell didn't burst in this case. These suggest that the cations ; Ca^{2+} or Mg^{2+} used here play the role(s) in keeping the structure of cell membrane of the protoplast, although only Ca^{2+} plays the additional role(s) on protoplast survival. During the culture of protoplast, it was observed that the volume of protoplast increased gradually, although many

of them ultimately bursted or released the cytoplasts. But no cell division was observed in the system used here.

Observations of the regeneration process of the cell wall by scanning electron microscope revealed that the early process was similar to that observed in higher plants; tobacco and grapevine (BURGRESS and LINSTEAD, 1976)(Figs. 9–16), although the complete regeneration of the cell wall didn't occur. BURGRESS and LINSTEAD (1977) reported in tobacco leaf protoplast that the formation of fibrous structure was completely suppressed for 2 days in the medium containing coumarin, but a partial recovery of the cell wall regeneration was observed after 4 days of culture. In *Closterium*, however, no recovery was observed even after 6 days of culture in the medium containing coumarin (Fig. 8A). They also suggested in tobacco that the coumarin might be used in prolonging the protoplast state. This may also apply to the case in *Closterium*, because the protoplasts, even after 3 days of culture with coumarin, maintain the ability to regenerate the cell wall (Fig. 8A). We suggest that the coumarin might be used in synchronizing the cell wall regeneration, because the cell wall regeneration seemed to be well synchronized after releasing of the coumarin in *Closterium*.

It is interesting that some differences of physiological nature between mt^+- and mt^--cells were observed: 1) protoplasts were derived in higher yield from mt^+-cells than from mt^--cells (Fig. 5, Table 1), 2) "postculture" was more effective in mt^--cells than in mt^+-cells (Fig. 5), 3) protoplasts from mt^--cells were more viable than mt^+-protoplasts after 6 days of culture (Figs. 6, 7A), 4) mt^--protoplasts were more viable at 3,000 lux than at 700 lux, which was the reverse nature of mt^+-protoplasts (Fig. 6), and 5) Calcofluor-detectable cell wall regeneration was inhibited in the dark for mt^--protoplasts (Fig. 8B), but not for mt^+-protoplasts (data not shown). These differences may be the reflection of the differences of the nature itself determined genetically of each mating type cell of heterothallic strains.

Some attempts are now in progress. Protoplast is being isolated from the gamete or zygote. Artificial zygote by protoplast fusion is being tried to make. And the more improved culture media of the protoplast are being searched.

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