Evaluation of Preparation Methods for Scanning Electron Microscopic Observation of Plant Protoplasts

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Received October 3, 1994

Abstract: Preparation methods for scanning electron microscopy (SEM) were studied with petunia and rice protoplasts. Fixation schedules to sustain protoplast size during alcohol dehydration were examined. When the different fixations were compared, the decrease in protoplast volume was observed to a variable extent during the dehydration process. The extent of volume decrease was reduced in order of glutaraldehyde, glutaraldehyde-osmium tetroxide, and glutaraldehyde-tannic acid-osmium tetroxide schedules. When fixed only with glutaraldehyde, the green color of the chloroplast in petunia leaf protoplasts was lost during alcohol dehydration. Well-defined scanning electron micrographs of petunia and rice protoplasts were obtained using a glutaraldehyde-tannic acid-osmium tetroxide schedule.

Key words: Dehydration, Fixation method, Petunia, Protoplast, Protoplast size, Rice, Scanning electron microscopy.

There are several reports on protoplast preparation for electron microscopy. However, little information is available with respect to the processing of protoplasts. Especially, no examination of the effects of fixative schedules was made in relation to the variation in protoplast volume during the alcohol dehydration process. By the 1960's, protoplast isolation was well established in dicot plants and later, starting around 1970, we began reporting on the isolation from monocot plants; rice and wheat. Consequently, using petunia and rice, an experiment was carried out to scrutinize the effects of fixation and dehydration on the morphology of plant protoplasts in order to clarify the involvement of the preparation procedures for scanning electron microscopic images.

Materials and Methods

Leaves of Petunia hybrida Vilm. (cv. Violet) plants grown in a greenhouse and mature seeds of Oryza sativa L. (cv. Nipponbare) were used to isolate protoplasts.

For rice callus induction:

Rice callus was obtained from the seeds on the induction medium (MS medium.supplemented with 10–6 M 2, 4-dichlorophenoxyacetic acid) using the method of Nakamura and Maeda[14]. Calli induced mainly from scutellum 30 days after incubation were subcultured on a medium similar to the induction medium and used for the experiment before a lapse of one month in the subculture.

For isolation of petunia and rice protoplasts:

The petunia protoplasts were prepared by incubating the stripped leaf pieces in an
Table 1. The conditions used for fixation of protoplasts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fixation procedure</th>
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</thead>
<tbody>
<tr>
<td>1, GA</td>
<td>2% GA for 2h at room temperature</td>
</tr>
<tr>
<td>2, GA and O₃O₄</td>
<td>2% GA for 2h at room temperature followed by 1% O₃O₄ for 1h at room temperature</td>
</tr>
<tr>
<td>3, GA</td>
<td>2% GA overnight at 4°C</td>
</tr>
<tr>
<td>4, GA, TA and O₃O₄</td>
<td>2% GA overnight at 4°C and 1% TA for 2h at room temperature followed by 1% O₃O₄ for 1h at room temperature</td>
</tr>
<tr>
<td>5, GA at serial conc.</td>
<td>0.5%, 1%, 1.5% and 3% GA for 1, 1, 2h and overnight at 4°C, respectively</td>
</tr>
<tr>
<td>6, GA at serial conc. and O₃O₄</td>
<td>0.5%, 1%, 1.5% and 3% GA for 1, 1, 2h and overnight at 4°C, respectively, followed by 1% O₃O₄ for 1h at room temperature</td>
</tr>
<tr>
<td>7, GA, TA and O₃O₄</td>
<td>A mixed solution of 2% GA and 1% TA for 2h at 4°C followed by 1% O₃O₄ for 1h at room temperature</td>
</tr>
</tbody>
</table>

Note. The fixation solution was supplemented with 0.4 M mannitol and 10 mM CaCl₂·2H₂O (pH 5.8). GA: glutaraldehyde, TA: tannic acid, O₃O₄: osmium tetroxide.

enzyme solution containing 0.2% (w/v) Macerozyme R-10 (Yakult Honsha, Japan), 2% (w/v) Cellulase Onozuka R-10 (Yakult Honsha, Japan), 10mM CaCl₂·2H₂O and 0.4M mannitol (pH 5.8). On the other hand, the rice calli were cut into small pieces and incubated in an enzyme solution containing 1% (w/v) Macerozyme R-10 (Yakult Honsha, Japan), 4% (w/v) Cellulase Onozuka RS (Yakult Honsha, Japan), 10mM CaCl₂·2H₂O and 0.4M mannitol (pH 5.8). After cell wall digestion on a 40–rpm shaker in the dark at 25°C during 4 h for petunia leaves and 6 h for the rice calli, each group of protoplasts was filtered through a nylon cloth (350 mesh) and collected by centrifugation at 100×g. The centrifugation time was 2 min for the petunia protoplasts and 5 min for the rice protoplasts. The pelleted protoplasts were washed three times at 100×g for 2 min each with a washing solution containing 10mM CaCl₂·2H₂O and 0.4M mannitol (pH 5.8).

**For estimation of protoplast size:**

The collected protoplasts were fixed in a constant concentration or serially increased concentrations of glutaraldehyde (GA) with or without 1% tannic acid (TA) at a different temperature and for a different duration, as shown in Table 1 and after that, washed with the washing solution described above. The fixed and washed protoplasts were adhered on small pieces of thoroughly washed coverglasses coated with 0.1% poly-l-lysine (MW 98,860; Sigma), and then dehydrated directly or after post-fixation with osmium tetroxide (O₃O₄) as shown in Table 1. In treatments 5 and 6, GA concentrations were gradually increased to higher levels. The flow diagram of the fixation series is shown in Fig. 1.

When the dehydration of the specimens was made through a graded ethanol schedule at intervals of 10 min, the diameter of the adhered protoplasts gradually decreased according to the increase in ethanol concentration because the protoplasts were attached at their relatively small, limited area to the surface of coverglass pieces. The diameter of protoplasts was estimated at each step of dehydration on a light microscope. Mean diameter was estimated from about 5 to 7 protoplasts at each step. The volume was calculated from the measured diameter as follows:

\[ V = (4/3)\pi \times (d/2)^3 = kd^3 \]

where \( V \) = volume of protoplast

\( \pi \) = the circular constant

\( d \) = protoplast's diameter

\( k \) = constant, \((4/3)\pi \times (1/2)^3\)

Protoplast volumes, which were calculated at various steps of dehydration, were finally represented as a percentage to the value at the beginning of dehydration as follows:

\[ P_s = \left( \frac{k(d_s)^3}{k(d_i)^3} \right) \times 100 \]

\[ = \left( \frac{(d_s)^3}{(d_i)^3} \right) \times 100 \]

where \( P_s \) = percentage at various steps

\( d_s \) = the diameter at various steps

\( d_i \) = the diameter at the beginning of dehydration

**For scanning electron microscopy (SEM):**

A critical point drying method was done
using isomyl acetate and carbon dioxide to dehydrate the specimens on coverglass pieces. The dehydrated specimens on coverglasses were mounted on a metal stub with double-stick tape, coated with gold under vacuum and then examined on a Hitachi scanning electron microscope (S-415) by operating at 25kV.

Results and Discussion

As shown in Table 1 and Fig. 1, various fixation conditions were used to determine their influence to the variation in volume of the protoplasts isolated from petunia and rice. The variation in the protoplast volume during the ethanol dehydration process is shown in Fig. 2, where the volume immediately after the fixation is expressed as 100%.

In petunia leaf protoplasts, the final volume after dehydration was decreased to about 54%, 65%, 65%, 79%, 80% and 93% in treatments 1, 3, 5, 6, 2 and 4, respectively (Fig. 2, A). It was of interest that the protoplast volume was nearly the same in treatments 3 and 5 where GA alone was used. The volume was also similar between treatments 2 and 6 where both GA and O₃O₄ were used.

It is well known that an aldehyde solution is excellent for the fixation of protein, but not for lipids, especially phospholipid\(^8\). Therefore, when the protoplasts were fixed in GA alone, not only did their volume gradually decrease, but also the green pigments of chloroplasts contained in the petunia leaf protoplasts were markedly reduced (Fig. 3). On the other hand, O₃O₄-fixation following GA maintained the green color at the end of dehydration (Fig. 4). That is to say, nevertheless the fixation with GA was performed for a longer time (overnight in treatments 3 and 5), the pigments of chloroplasts leaked out because of insufficient fixation of lipids in chloroplasts. Several reports have mentioned a leakage of lipids from a specimen during fixation and dehydration\(^3,11,12,16\). It was demonstrated that only about 30% of the chlorophyll content in the isolated spinach chloroplasts remained even in the materials fixed by O₃O₄\(^5\).

Concerning the tannin-osmium method for SEM, Futaeaku\(^9\) has indicated that TA allows soluble proteins and polypeptides to deposit in the treated cells and accelerates the

![Flow diagram of fixation and dehydration for the preparation of protoplasts.](image)

![Graph showing volume (%) of protoplasts of *Petunia hybrida* Vilm. cv. Violet (A) and *Oryza sativa* L. cv. Nipponbare (B) during dehydration. The volume of fixed protoplasts at the time starting dehydration is expressed as 100%.](image)
stabilization by covalent bonds of these substances, which are susceptible in the cells fixed with aldehyde alone. Furthermore, it has been mentioned that a catechol base in TA behaves to make chelate linkage between metal cation and $O_2O_4$ through its high affinity. This opinion supports our result that the decrease in volume by the application of TA (treatments 4 and 7) is less than that by other methods without TA. In addition, it is notable that TA cross-links a variety of proteins but the linkage is in competition with GA$^6$.

In the case of rice callus protoplasts lacking in well-developed chloroplasts, the decrease in volume in treatment 4 was slight, as shown in Fig. 2 (B), maintaining the value at about 90%. In treatment 7, the value was 88%. However, the value of rice protoplasts in treatment 3 (GA overnight) was 54%, which was 11% lower than that of petunia protoplasts in
the same treatment and similar to petunia protoplasts in treatment 1, where GA treatment was only for 2 h. Consequently, it is obvious that it is difficult to retain the morphology of rice protoplast under treatment with GA alone. This probably resulted from a higher proportion of soluble proteins and polypeptides in the contents of rice protoplasts than those of petunia protoplasts.

Figures 5 and 6 show scanning electron micrographs of well-preserved protoplasts of petunia and rice, respectively. They were fixed by the schedule of GA, TA and $\text{O}_2\text{O}_4$.

It is clear from these figures that the diameter of petunia protoplasts is about two-fold the value of rice protoplasts. Petunia leaf protoplasts are better identified by the presence of chloroplasts lying beneath the plasma membrane. The presence of chloroplasts was more distinctly seen in the case of 0.5M than 0.4M of mannitol in the fixative solutions, because of the slight shrinkage of protoplasts in the higher osmolarity. The surface of petunia protoplasts was mostly smooth in its appearance but the smooth surfaced protoplasts of rice were fewer than the petunia protoplasts (Fig. 6). However, special appendixes, such as fibrillar materials and fragments of remaining cell wall, were not found on the surface of petunia and rice protoplasts. Scanning electron microscope images of well-preserved protoplasts were observable in all fixation treatments but their frequency was remarkably high in the appropriate fixation schedules, by which a lower decrease was attained in the protoplast volume.

Serious damage occurred in some protoplasts affecting their shape in various ways during the dehydration process even after a deliberately designed fixation. The protoplasts shrank and collapsed gradually in 30% ethanol and suddenly in absolute ethanol. The poorly fixed protoplasts underwent more intensive damage during dehydration than the well-fixed protoplasts. Moreover, the contraction of a specimen may have resulted from the process of critical point drying. A revised method using $t$-butyl alcohol was developed with less influence on specimen$^8$, and further SEM equipped with a cryo-system is recommended to avoid a chemical preparation of specimens. These methods will be useful to observe in detail the ultrastructure of plant protoplasts.

In conclusion, this paper showed that the variation in the shape of protoplasts is detectable during alcohol dehydration under light microscope observations. This finding would be helpful in optimizing the preparation method of plant protoplasts for SEM observation.

References

* In Japanese.