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Re-evaluation of FDA as a Vital Staining for Plant Cells

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植物細胞에 대한 生體染色劑로서의 FDA의 再評價

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Summary

The main purpose of this investigation was to evaluate the accurate use of FDA(Fluorescein diacetate) and Uranin(soluble fluorescein). Which were well known vital staining agent to testing the vitelity of living cell, compared by the response of urea permeability, cytoplasmic streaming and fluorecing quality after treatment of two kind of metabolic inhibitor in the protoplasm of adaxial epidermal cells of the Allium cepa bulb scale and sub-epidermal cells of the *Pisum sativum*.

The results obtained are as follows :

1. The speed of cytoplasmic streaming measured with onion epidermal cells in control was about 7 μm/sec. and did not change for 2 hours, whereas, for all concentration used, both metabolic inhibitors decreased streaming speed as time elapsed. Especially, cells treated with DNP for 120 min, almost stopped cytoplasmic streaming in 2 hours.
2. Brightness of fluorescence by FDA or Uranin staining could not be distingished by naked eyes under the microscopic observation even after inhibitor treatment. From the data measured by micro-fluometer(Zeiss epifluorescent microsoope), on the other hand, the cells stained with FDA exhibited brighter fluorescence than with uranin by 7 times although concentration of uranin in staining solution was 10 times higher than FDA.
3. As expected, plasmolysis itself was not affected with the pretreatment of FDA and Uranin : however the permeability constant increased by almost 100% and by 35% with pretreatment of FDA and Uranin respectively.

In conclustion, FDA seems not to be good to identify metabolically active cells despite its good performance for teating dead cells, However, vital stainings are not highly recommended to screening metabolically active cells.

Therefore new method should be developed for evaluating metabolic status of isolated protoplasts.

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Introduction

Test of viability of cells or isolated protoplasts is essential for protoplast culture, protoplast fusion and direct transfer of genes to protoplasts (3).

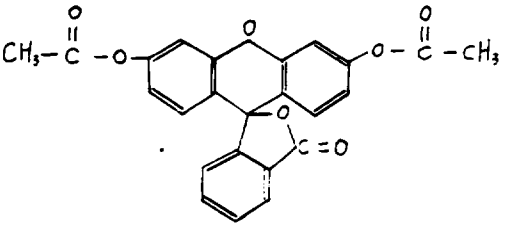
Vital staining is one of very useful methods which can test the viability of cells. Various stains are available for testing cell viability depending on plant materials and experimental purpose(2, 8, 10).

Among various vital stains, fluorescein diacetate (Fig. 1a) has been most common stain for identifying living protoplasts because of its easiness for application and relatively quick reaction to living cells under fluorescent microscope (6, 11). A fluorescein, product of cytoplasmic esterase activity on FDA, can accumulate in living cells but not in dead cell (6).

The integrity of isolated protoplasts, however, is often questioned because of their poor developmental behavior even though their viability have been tested by FDA. Also, stains for detection of fused protoplasts (heterokaryons) can damage protoplasts when stains remained in the cell for long period (4).

Therefore, the principal purpose of this experiment was to investigate whether FDA can screen the functionally (or metabolically) intact cells or protoplasts. Application of vital stains to cells of which vitality are decreasing by the treatment of metabolic inhibitors has not been studied. Also, possible injury of cells by FDA staining was tested because FDA is frequently dissolved in acetone which may cause damage on the plasma membrane.

a) Fluorecein diacetate(FDA)



b) Uranin(Fluorecein disodium)

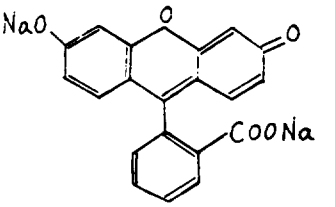


Fig. 1. Molecular structure of FDA and Uranin

Uranin (Fig. 1b), another fluorescein stain, was chosen for the comparison with FDA, because uranin, soluble in water, is the fluorescent product of esterase activity on FDA. Therefore, damage to cells or protoplast by organic solvent can be avoided.

Materials and Methods

1. Plant materials

Organically grown onions (*Allium cepa*) were purchased from the Co-op market (SAP, Cleveland Ave. St. Paul, MN). Adaxial epidermal sections of the 3rd scale from the onion bulbs were prepared after Stadelmann (9).

Peas (*Pisum sativum*) were grown in the pots under the controlled environment after Lee-Stadelmann (7). From 4-week-old plants, peeled stem sections of the 1 st internode were prepared.

2. Chemicals

Sodium azide (NaN_3). 2, 4-dinitrophenol (DNP), fluorescein diacetate (FDA) were from Sigma. Uranin (disodium fluorescein) was purchased from Hartman-Leddon Co.

3. Cytoplasmic streaming

Cytoplasmic streaming was observed as an indicator of metabolic activity as well as viability of cells (5, 9).

To decrease metabolic activity of the cell, azide and DNP, dissolved in spring water (Glenwood, Minneapolis, MN; about pH 8), were used as metabolic inhibitors for this experiment. Azide is an inhibitor of cytochrome C oxidase and DNP is an uncoupler for electron transport system of mitochondria.

Speed of cytoplasmic streaming was measured by recording the movement of small particles in onion cells under the microscope (8). Speed of cytoplasmic streaming was monitored for 2 hrs. For treated cells, metabolic inhibitor(10mM azide or 0.1mM DNP) was treated for 10, 30, 60, 90 and 120 min before the measurment of cytoplasmic streaming.

4. Vital staining

a) FDA

FDA stock solution (5㎎ FDA/1㎖ acetone) was diluted 50 times with spring water just before use. After a section was mounted on a drop of diluted FDA solution on a slide glass, cells were observed under the Zeiss epifluorescent microscope.

b) Uranin

Uranin stock solution (0.1%) was diluted 10 times with phosphate buffer (pH 8.0). Sections were stained for 20 min. in the buffered uranin solution. After sections were washed with the same phosphate buffer for 10 min, cells were observed under the Zeiss epifluorescent microscope.

5. Micro fluometry

After treatment with metabolic inhibitors for 30, 60, 90 and 120 min, onion sections were floated in spring water. Sections were, then, stained with FDA or uranin for 10 min. respectively. Sections stained with uranin was washed in spring water for 10 min. Transmittance of fluorescence intensity of cells was measured by a fluorometer attached to Zeiss epifluorescent microscope (flow cytometry). Plant tissue was excited by blue light (475nm) from UV lamp. Emitted fluorescence was measured by fluorometer. For each preparation, 10 readings were made from 3 sections.

6. Measurement of urea permeability

Urea permeability was measured and calculated after Stadelmann (9). The permeability was measured to test the intactness of the plasma membrane after staining.

Sections were plasmolyzed in series of mannitol solutions (0.1, 0.3, 0.5, and 0.7M) for 20 min at each concentration step. When uranin or FDA was applied, sections were stained for 10 min before starting plasmolysis procedure.

After complete plasmolysis, a section was mounted on the perfusion chambers. For both plant materials, the epidermal side was faced up. Three cells with cylindrical shape were chosen for the measurement. Changes in protoplast length was recorded under the microscope with 10 min interval as urea solution (0.7M) flew through the chamber with constant flow rate by using a peristaltic pump. Protoplast expansion was recorded for about 2hrs.

1. Measurement of cytoplasmic streaming

The speed of cytoplasmic streaming measured with onion epidermal cells was about 7μm/sec. The speed of control cell actually did not change for 2 hrs (Fig. 2 and 3). For all concentration used, both metabolic inhibitors decreased streaming speed as time elapesd. As increase in treatment period, streaming rate decreased in proportional to the treatment period. Cells treated with DNP for 120 min almost stopped cytoplasmic streaming in 2 hrs.

2. Microphotography

Cells (control and treated) were stained

Results

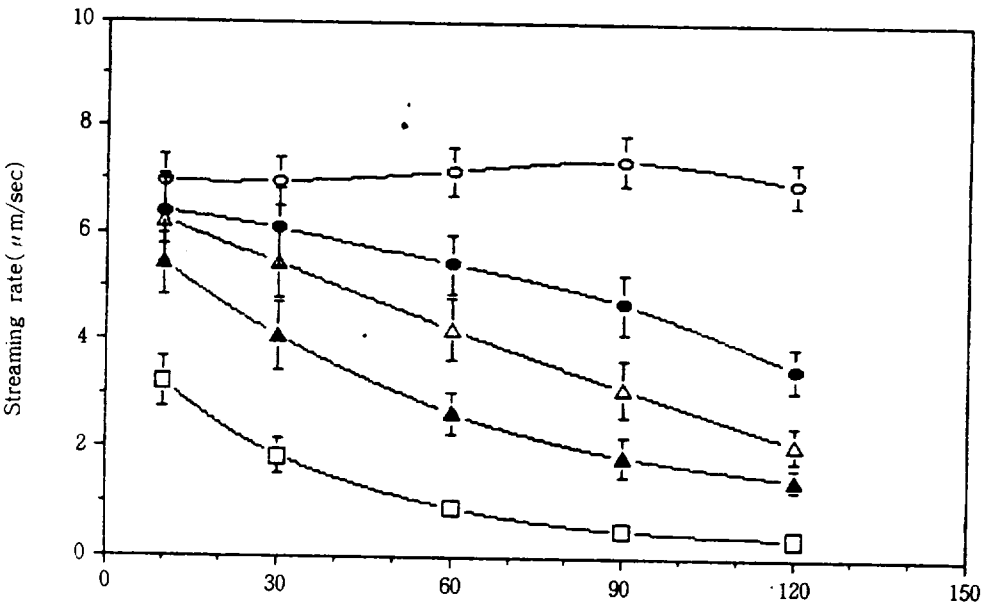


Fig. 2. Effect of 2, 4-Dinitrophenol (DNP) on Cytoplasmic streaming in epidermal cell of *Allium cepa* ○—○ : control ●—●DNP 30min. △—△ : DNP 60min. ▲—▲ : DNP 90min □—□DNP 120min.

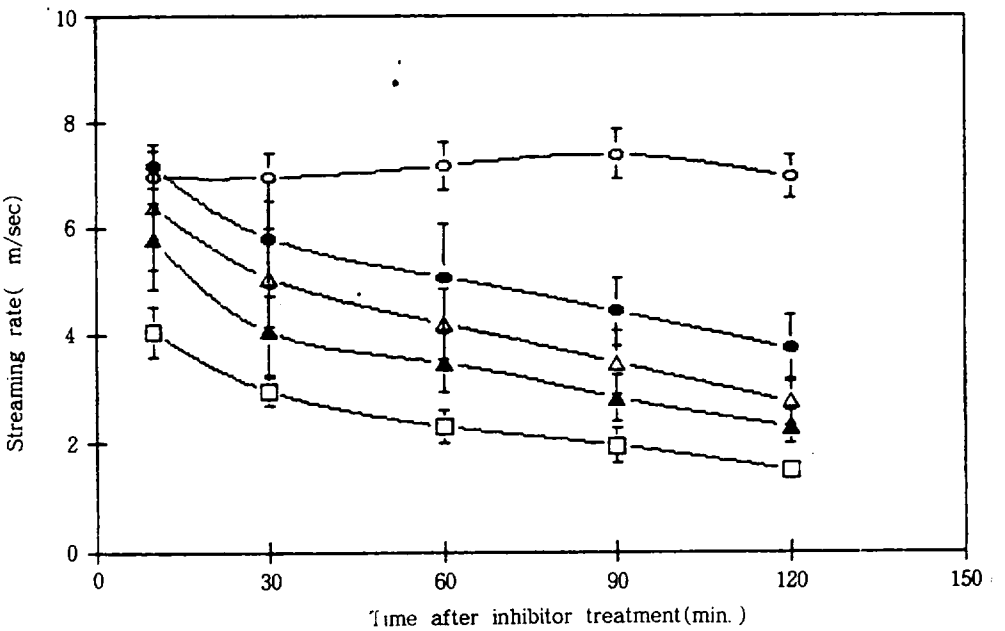


Fig. 3. Effect of NaN_3 on cytoplasmic streaming in epidermal cell of *Allium cepa*
○−○ : control ●−● : NaN_3 30min. △−△ : NaN_3 60min. ▲−▲ : NaN_3 90min □−□ NaN_3 120min.

with both FDA and uranin. Brightness of fluorescence by FDA or uranin staining could not be distinguished by naked eyes even after inhibitor treatments. Only cells stained with uranin showed dull fluorescence after treatment with DNP for 120 min.

3. Fluorescence intensity

Cells from both plants showed good fluorescence by either FDA or uranin. Cells stained with FDA exhibited brighter fluorescence than with uranin by 7 times (Table 1) though concentration of uranin in

Table 1. Changes in relative transmittance of fluorescence from epidermal cells of *Allium cepa* by FDA or uranin staining.

Staining		FDA				Uranin			
Period of inhibitor treatment	(min.)	30	60	90	120	30	60	90	120
Control		100	100	100	100	100	100	100	100
DNP		86.3	83.9	68.0	54.1	77.8	75.3	67.0	53.5
Azide		88.1	74.6	73.1	66.5	94.1	84.9	71.8	68.5

*Fluoresecent intensity of FDA was about 7 times higher than that of uranin (14.5% of FDA fluorescence).
Metabolic inhibitors were treated for 30, 60, 90, and 120 min before staining.
Fluorescence for control cells was not changed significantly for 2hr period.

staining solution was 10 times higher than FDA. After 2hrs since section preparation, fluorescence of cells by either stain was as good as of cells immediately prepared.

After treatment with inhibitors, fluorescence intensity decreased gradually when cells were stained with uranin or FDA. Fluorescence decreased to 50% after treatment of DNP for 120 min.

4. Urea permeability

Table 2. Comparison of urea permebility constants of subepidermal cells from *Pisum sativum* and *Allium cepa* after pretreatment with FDA and uranin for 120 min.

Treatment	Allium cepa	Pisum sativum
Control	3.82±0.54(11)	3.36±0.51(10)
Uranin	5.13±0.91(12)	4.53±0.51(11)
FDA	1.92±1.16(9)	5.86±0.52(9)

Numbers in parenthesis indicate number of cells measured.

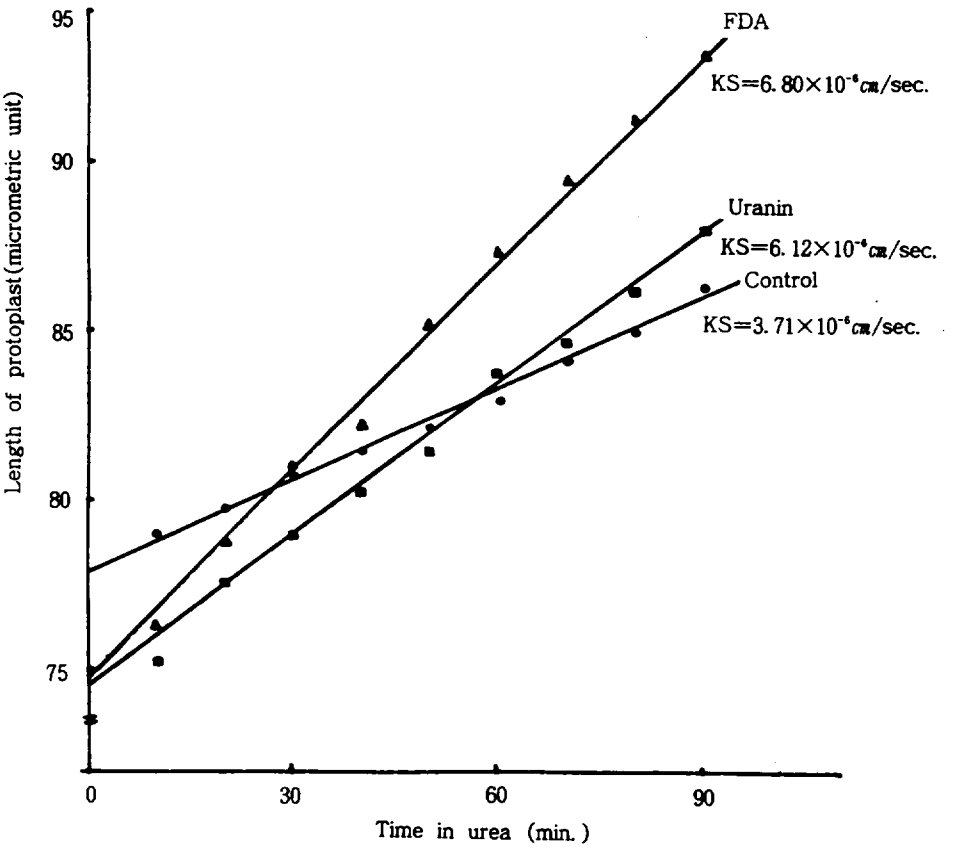


Fig. 4. Time course of sample graph for urea permeability in epidermal cell of *Allium cepa*

As expected, plasmolysis itself was not affected with the pretreatment of FDA or uranin. However, significant increase in urea permeability was detected with the pretreatment of either stain (Table 2, Fig. 4, 5). The permeability constant increased by almost 100% and by 35% with pretreatment of FDA and uranin respectively.

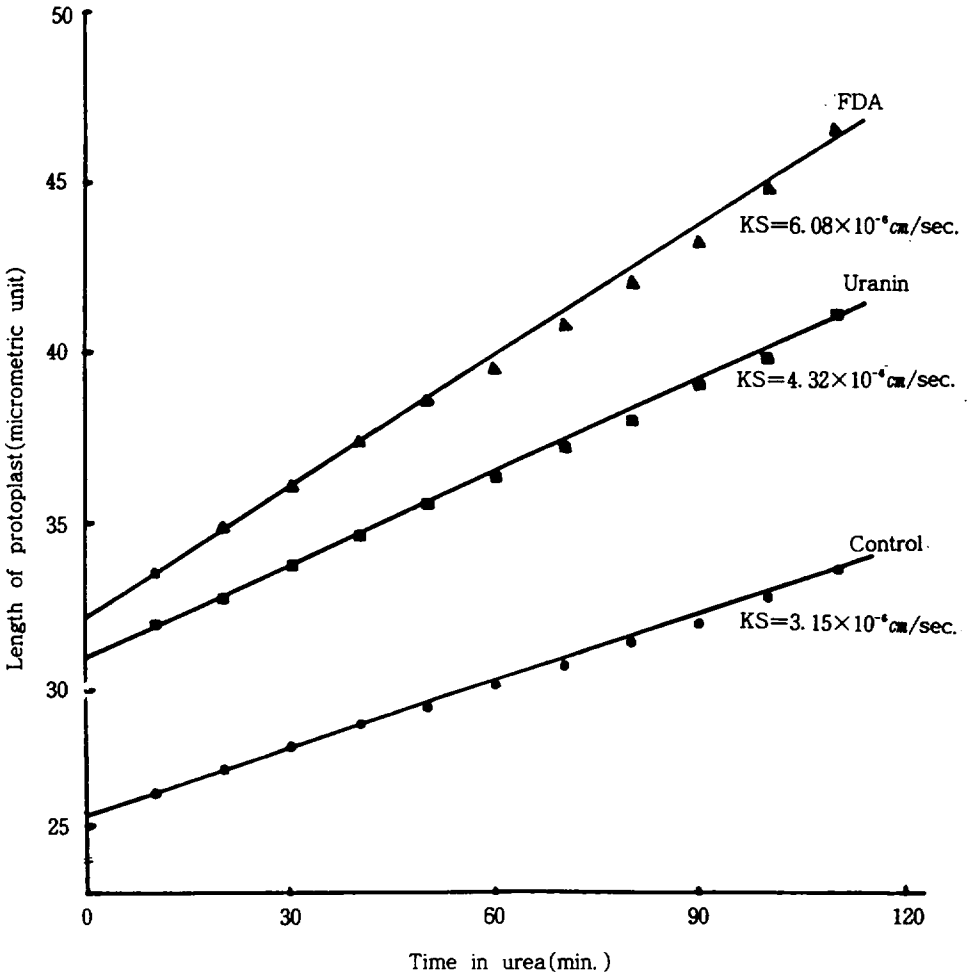


Fig. 5. Time course of sample graph for urea permeability in sub-epidermal cell of *Pisum sativum*

Discussion

Since cytoplasmic streaming can be used as an indicator or metabolic activity (5), decrease in speed of cytoplasmic streaming by the inhibitors (Fig. 2, 3) clearly showed diminishing cell vitality. Decrease in cytoplasmic streaming is due to low amount of cytoplasmic ATP which is required for this

movement because the inhibitors prevent ATP synthesis in mitochondria (1).

Decreasing fluorochromatic reaction of cells to FDA and uranin was relatively proportional to cell vitality (i.e., cytoplasmic streaming) as shown in Tabel 1. However, as mentioned before this decrease in fluorescence was hardly recognized with naked eyes under the microscope. This suggests that these vital stains could not be good clues to present the metabolic intactness of cells even though they are good for detecting dead cells. Recently, Arora and Palta (1988) showed that freezing injured cells which lose 85% of their Ca^{++} in the membrane could exhibit fluorochromatic reaction to FDA and plasmolysis.

Therefore, cells can be stained with FDA or uranin if they can keep membrane integrity to certain extent even their metabolic activity decreases. In contrast to drastic decrease in cytoplasmic streaming of cells after inhibitor treatment, less decrease of fluorescence can be explained by their relationship with cytoplasmic ATP level. That is, ATP is absolutely required for cytoplasmic streaming, however, passive accumulation of uranin and esterase activity for converting FDA to soluble fluorescein do not require ATP.

Decrease in amount of uranin or FDA trapped inside the protoplasm by low vital cells in comparion to healthy cells may be due to loss of intactness of the plasma membrane, i.e., leaking of accumulated fluorescein, which is not freely permeable across the membrane, to external solution.

Since passive permeability (urea) increased by staining without other treatments which can alter lipid portions of the plasma membrane, it may indicate that damage of cell membrane (9). The membrane damage can be resulted from two possible reasons : i) harmful effect of organic solvent (i.e., acetone) for dissolving a vital stain (3) and ii). accumulation of fluorescein inside the protoplasm.

Because of tremendous increase of urea permeability by FDA compared to uranin, harmful effect of acetone on the plasma membrane is very possible. However, presence of relatively high amount of fluorescein, product of cytoplasmic esterase activity on FDA, cannot be excluded completely for the membrane damage. Relatively small amount of uranin is accumulated inside the protoplasm.

In conclusion, FDA seems not to be good to identify metabolically active cells despite its good performance for testing dead cells. Rather uranin seems to be better for identifying functionally intact living cells under microscope with nakey eyes. However, vital stainings are not highly recommended to screening metabolically active cells. Therefore, we do believe that new method should be developed for evaluating metabolic status of isolated protoplasts. Attention should be also paid when cells have to be maintained for long period after staining, because staining might damage the living cells.

국 문 초 록

살아있는 세포의 활성을 검정하는데 널리 이용되고 있는 FDA와 Uranin에 대한 Urea의 투과성과 세포질 유동 그리고 세포 대사 억제물질 처리 후의 발광 정도를 조사하여 공시한 2가지 약제(FDA, Uranin)의 이용에 대한 정확한 평가를 하기 위하여 양파의 표피 조직과 완두콩의 2차 표피 조직을 대상으로 본 실험을 실시하였으며, 얻어진 결과는 다음과 같다.

1. 양파의 표피 조직을 이용한 표준 원형질 유동 속도는 7㎛/sec. 이며 2시간이 경과한 후에도 속도의 변화는 없었지만, 식물 세포 대사 억제로서의 DNP와 NaN 처리에서는 시간이 경과함에 따라 모든 농도에서의 유동 속도가 감소하였다. 특히 DNP 2시간 처리후 120분이 경과되면 모든 원형질 유동이 정지됨을 볼 수 있었다.

2. FDA나 Uranin 염색에 의한 형광의 밝기 정도는 대사 억제물질이 처리된 후에도 현미경 하에서 육안으로 뚜렷이 구별할 수 없었다. 그러나 한편 형광량 측정 현미경으로 측정된 수치는 FDA가 Uranin과 비교할 때 7배나 더 밝은 것으로 나타났는데 염색약제로 회석되는 배율은 Uranin이 FDA보다 10배 높은 농도의 용액이다.

3. FDA와 Urnin의 전처리에서는 원형질 분리 현상이 기대했던 바와 같이 어떠한 영향도 주지 않았으나, Urea 투과율을 비교해보면 FDA가 100%일 때 Urnin이 35%의 비율로 투과됨을 알 수 있었다.

결론적으로 FDA는 죽은 세포를 검정하는데 적합한 약제일수는 있지만 대사적으로 활성있는 세포의 활성을 조사하는데는 부적합함을 알 수 있다. 따라서 생체 염색 방법은 식물세포의 체내 대사의 활성을 검정하는데 썩 좋은 방법이라고 할 수 없기 때문에 특히 분리된 원형질체의 활성을 검정하는데는 앞으로 누구나가 신뢰할 수 있는 새로운 기술이 요망되는 바이다.

References

1. Arora, R. and Palta, J. P. (1988). *In Vitro* perturbation of membrane-associated by freeze-thaw stress in onion bulb cells Plant Physiol 87 : 622-628.
2. Fisher, J. M. C., Peterson, C. A. and Bols, N. C. (1985). A new fluorescent test for cell viability using calcoflour white M2R. Stain Technol. 60(2) : 69-79.
3. Galbraith, D. W. and Galbraith, J. E. C. (1979). A method for identification of fusion of plant protoplasts derived from tissue cultures. Z. Pflanzenphysiol. 93 : 149-158.
4. Glimeiius, K. (1987). Enrichment of somatic hybrids with flow cytometry.
5. Kamiya, N. and Kuroda, K. (1973). Dynamics of cytoplasmic streaming in a plant cell. Biorheology 10 : 179-187.
6. Larkin, P. J. (1976). Purification and viability determination of platn protoplasts. Plannt 128 : 213-216.
7. Lee, O. Y. (1975). Studies on the effect of water stress on the proto plasm of *Pisum sativum* Subepidermal stem cells. Thesis of PhD. Univ. of Minnesota U. S. A.
8. Palta, J. P., Levitt, J., and Stadelmann, Ed. J. (1978). Plant viability assay. Crybiology 15 : 249-255.
9. Stadelmann, Ed. J. (1966). Evaluation

of turgidity, plasmolysis, and deplasmolysis of plant cells. In : Methods in cell physiology (Prescott, D. M. ed.), Vol. 11, pp. 143-251.

10. Stadelmann, Ed. J. and Kinzedl, H. (1972). Vital staining of plant cells. In : Methods in cell physiology (Prescott, D. M. ed.), Vol. V, pp. 325-375.

11. Widholm, J. M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47 : 189-194.