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Relationship between Cell Coagulation in a Suspension Culture and Cell Division in a Static Culture for Grape Protoplasts

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In an attempt to apply protoplast culture in breeding, it is particularly important to culture singly isolated protoplasts in a static culture, that is, gel entrapped protoplasts. As reported earlier[1], we succeeded in developing singly isolated protoplasts of grape (Kosyu) to callus in static culture. However, the established culture condition was not adequate for other varieties of grape. In most cases, specifically appropriate culture conditions are required for each variety. Identifying such conditions for each variety of grape would be a formidable task, because the viability of protoplasts depends not only on culture conditions, but also on the source of tissues and the method used for protoplast isolation. A fairly long time would also be required to judge the result of cultures. Recently, however, an interesting relationship between the number and morphology of flocks formed in suspension cultures, that is, liquid media containing protoplasts, and the frequencies of cell divisions in static cultures was observed. In this report, the relationship was investigated in grape protoplasts.

The protoplasts used were prepared from a suspension culture derived from the shoot of *Vitis vinifera* L. cv. Kosyu according to previously reported methods[1]. Unless otherwise stated, all the protoplasts used were prepared from the suspension in the late logarithmic phase. For suspension culture, protoplasts were cultured in a basal medium (pH 6.0) supplemented with 3% sucrose, 7% mannitol 10 mg/*l* NAA and 0.01 mg/*l* zeatin in 0.25 m*l* per well (ϕ 1.8 cm) on a 24-well plate (Corning, New York) by adjusting the initial concentration of about 2×10^5 cells/m*l*. Cultures were then sealed with parafilm and incubated at 27°C in the dark, unless otherwise specified. The number of protoplasts was counted under a microscope on a Fucks-Rosenthal chamber. The static culture was performed as reported previously[1]. A cluster consisting of more than four cells, with the longest span of more than 90 μm, formed at 2-3 days after the inoculation, was defined as a flock. The size and numbers of flocks formed were measured by an objectometer and counting chamber, respectively. Cell wall regeneration within a flock was detected by fluorescence staining of cellulose by Calcofluor White M2R[2,3] (Sigma) at 365 nm for excitation and 410 nm for observation using an ORYMPUS FT-2 fluorescence microscope.

Flock formation was observed after about 2 days culture in a suspension culture of protoplasts derived from Kosyu callus, as shown in **Fig. 1**. Flock formation occurred concomitantly with cell wall regeneration, but preceded cell division. The regenerated cell wall was confirmed by fluorescence. Addition of 2, 6-dichlorobenzonitrile (Aldrich), a known inhibitor of cellulose synthesis[4,5], to the culture inhibited flock formation at a concentration of more than 0.01%, but protoplasts remained unchanged. Under these conditions, fluorescence originating from staining of cellulose by

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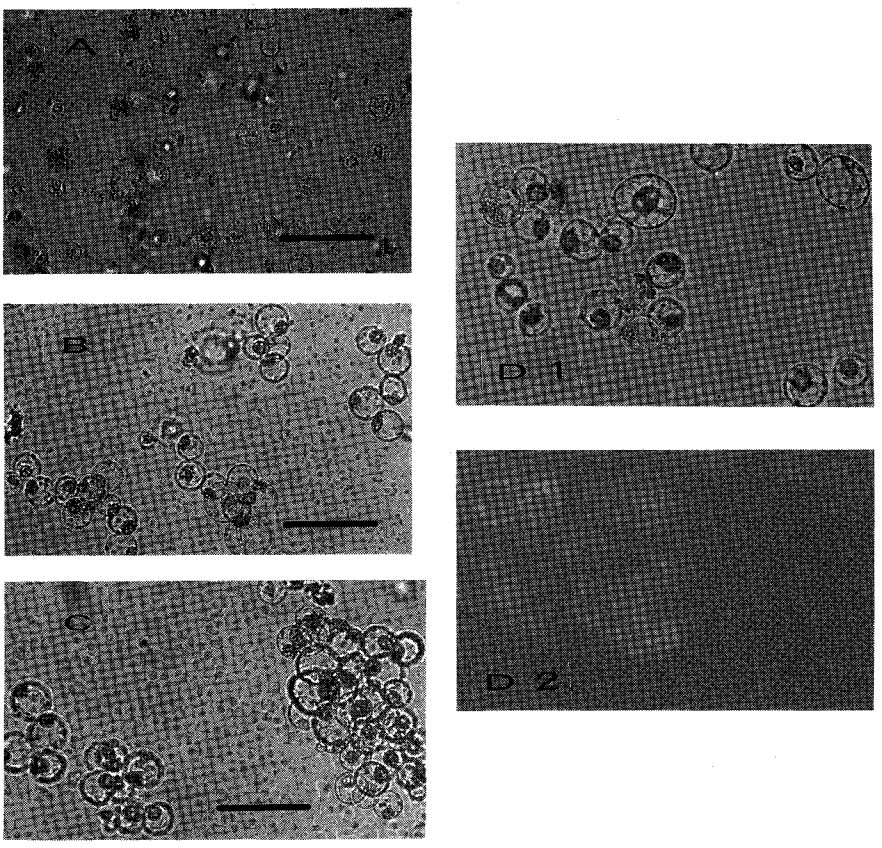

Fig. 1 Flock formation and cell wall regeneration in suspension culture of protoplasts from the grape "Kosyu".
Culture time (day) A : 1 day B : 2 days C : 3 days D : 2 days. A, B, and C were photographed with an optical microscope. D was photographed with both an optical microscope (D1) and a fluorescence one (D2) after staining with 0.1% Calcofluor White followed by washing several times with pH 6.0 MES buffer containing 0.5 M mannitol. The culture conditions were described in the text. (MS used as a basal medium).
Bar=100 μm.

Calcofluor White could not be observed. Thus, flock formation appears to be closely related to cell wall regeneration. Flock formation could also not be observed under conditions lowering the viability of protoplasts, as follows: non-nutrient medium (a solution such as MES buffer containing mannitol), unsuitable culture temperature (4, 10, and 45°C), UV irradiation (30 min. under UV light), or addition of Antimycin A (3.6×10^{-5} M).

Effects of various factors were examined as follows.

1) Cultivation stage of the callus. As shown in **Fig. 2**, callus derived from the logarithmic phase was the most suitable material for obtaining viable protoplasts. This growth stage of the callus also led to the maximum frequency of cell division in the static culture.

2) Cell density. **Fig. 3** shows the relation between protoplast density and the time course of flock formation. Each culture attained the maximum number of flocks after 3-4 days. In cultures with high protoplast density, the apparent number of flocks increased due to neighboring between the protoplasts and then decreased owing to interactions between the flocks.

3) Plant hormones and pH. Effects of phytohormones were investigated in suspension culture with NAA and zeatin, which were applied in the static culture[1]. Among samples tested under

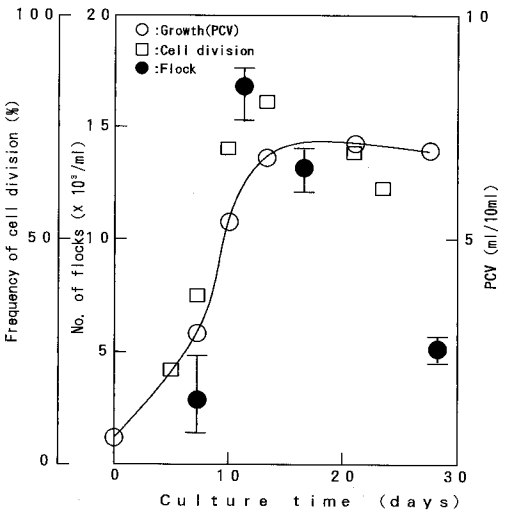

Fig. 2 Effects of culture stages of callus used as a source of protoplasts on flock formation in suspension culture and cell division in static culture.
Protoplasts were isolated from calluses at various culture times (days) and were cultured for 4 and 14 days in suspension and static cultures, respectively.
The numbers of protoplasts were adjusted to initial concentrations of about 1×10^5 and 2×10^5 cell/m*l* for static and suspension cultures, respectively. Vertical bars indicate range of values of formed flocks in suspension cultures. (●: mean value. $n=6$)
The frequency of cell division shown was the mean value of 9 arbitrarily chosen points of 0.5 cm^2 per dish for 3 replicates in static culture. Frequency of cell division: No. of cells divided more than once/No. of cells inoculated initially$\times100$ (%). Activated charcoal (0.05%) was added to the static cultures.
The growth of callus used as protoplast sources was estimated by packed cell volume (PCV) obtained by centrifugation at $150\times g$ for 5 min. from 10 m*l* of the culture.

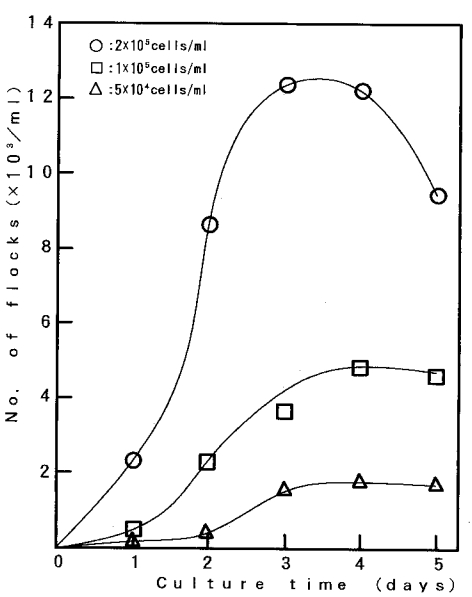

Fig. 3 Relation between the protoplasts density and time course on flock formation.
Each value was the mean of 4 measurements.
Source of protoplasts: Callus cells obtained from 17 days suspension in MS.

different conditions, *i. e.*, NAA (0, 1, 10, and 100 mg/*l*) and zeatin (0, 0.01, 0.1, and 1 mg/*l*), a combination of 10 mg/*l* NAA and 0.01 mg/*l* zeatin gave the most satisfactory result (14×10^3 flocks/m*l*), and also led to optimum cell division in the static culture, as previously reported[1]. Among the different pH conditions (5, 6, and 7) tested, pH 6.0 was found to be the optimum pH for the both culture methods.

4) Basal medium. Effects of various kinds of basal media on flock formation were investigated, and the frequency of cell division was examined in static culture using protoplasts prepared at the same time. As shown in **Fig. 4**, the most suitable condition was obtained with MS[6] without activated charcoal while the poorest results were found with white[7] or N'67[8]. However, even when using MS, which gave the maximum value, the division rate was 22%, which is less than satisfactory. As reported earlier[1], addition of activated charcoal was required for callus formation. Although cell division was greatly increased in static culture in MS with activated charcoal, the flock number decreased in suspension culture with activated charcoal. This result indicates that the relationship between cell division in static culture and flock formation in suspension culture

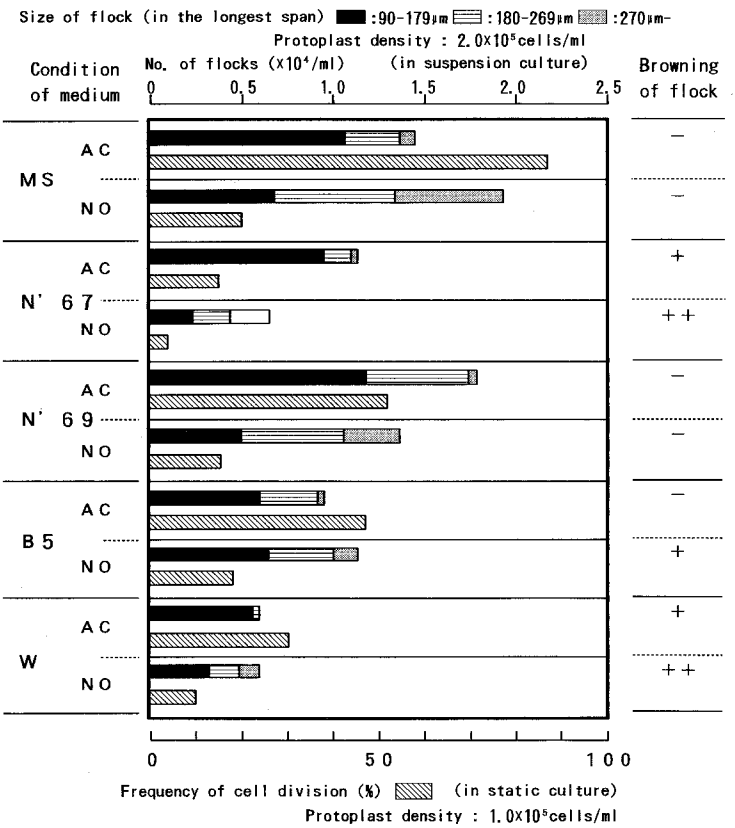

Fig. 4 Relation between the numbers and sizes of flocks formed in suspension culture and the frequency of cell division in static culture with various conditions for the protoplasts. Abbreviations were used as follows. AC: Activated charcoal (0.05%, w/v) was added to both the suspension and static media. NO: No addition of activated charcoal. (++): Flocks accompanied by browning, (+): Turned brown within a few days after inoculation, (−): No browning within 10 days after inoculation.
Source of protoplasts: callus cultured for 14 days in MS.
Culture time for protoplasts: 4 days for suspension cultures; 14 days for static cultures.
Each number of flocks formed is the average of six measurements.
Other conditions are given in **Fig. 2**.

cannot be discussed merely in terms of number of flocks formd. Similar results occurred between the media containing activated charcoal, such as MS and N'69[9], or N'69 and B5[10]. Namely, a positive relation between the frequency of flock formation and rate of cell division could not necessarily be confirmed among the basal media investigated.

5) Morphology of flocks formed. **Fig. 4** also shows that the proportion of small-size flocks increased in MS containing activated charcoal. In particular, many isolated deformed (cell wall regenerated) cells could also be found in MS containing activated charcoal, but very few of such cells were found in MS without it. It is thought that, under appropriate culture conditions, protoplasts proceeded with flock formation and accompanying cell wall regeneration, but immediately entered the next growth stage, which consequently weakened the progress of flock formation. Thus, the judgement of optimal static culture conditions requires consideration of both the results of frequency of flock formation and size of the flocks formed.

While browning of formed flocks was noted in some media, as shown in **Fig. 4**, browning was not induced even after one month in MS containing activated charcoal. Ordinally, browning must be avoided because it leads to the arrest of cell growth, and alternative conditions must be prepared for further growth[11].

Swelling of each cell was frequently seen in the absence of activated charcoal. Cell divisions were also rare under these conditions in static culture.

Using the results obtained above, the phenomenon of flock formation in suspension cultures, which was considered to be closely related to the viability of the protoplast itself, can be adopted as criteria, at least in basic principle, by which to judge the quality of static culture conditions. The following are four requirements necessary for optimal static culture: 1) Flock formation can be confirmed; 2) A high ratio of small sized flocks and the appearance of isolated deformed-cells can be confirmed; 3) Non browning of flocks formed; 4) Non swelling of protoplasts. By considering these requisites, the superior condition in a given basal medium under investigation for static culture could easily be determined. It will also be necessary to explore further to discover if the phenomena described above may be applied to other plant species.

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