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武庫川女子大紀要（自然科学）

Isolation and Culture of Protoplasts from *Gymnocalycium baldianum*(speg.)Speg. (Hikagyoku)Callus Cultures

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Isolation and culture of protoplasts from *Gymnocalycium baldianum*(Hikagyoku)callus cultures were examined. The peeling treatment of fruit largely excluded contamination during the induction and culture of callus, leading to successful formation of callus. Callus was induced effectively from the flesh using MS solid medium containing 0.01μM of kinetin and 0.01μM of 2, 4-dichlorophenoxyacetic acid(2, 4-D). The callus growth in the medium was largely promoted without causing a browning of the callus in the subculture. In preparation of protoplasts, the enzyme solution gave a high yield of protoplasts. Cell division into 2 cells was observed after 24 hours of culture, then micro colonies formed after 7 days, followed by colony formation within 2 weeks.

Introduction

Parenchyma may be rather specialized as a water-storage tissue. Many succulent plants such as the Cactaceae contain in the photosynthetic organs chlorophyll-free particularly large size and usually with thin walls.

In this study we induced callus tissues from the fruit of *Gymnocalycium baldianum*(Speg.) Speg., isolated protoplasts from the subcultured callus, and then cultured the protoplasts.

Materials and Methods

Induction and culture of callus

Fruit were collected from *G. baldianum*. After washing with running tap water, the fruit were surface-sterilized with 70% ethanol for 30 sec, further sterilized with 1% sodium hypochlorite aqueous containing a few drops Tween 80 for 5 min, and then rinsed three times in sterilized distilled water.

The peeled flesh was inoculated Murashige and Skoog medium(MS medium)[1]solidified by 0.8% agar, whose pH was adjusted to 5.8, and cultured for callus induction at 25℃ under fluorescence illumination of about 2,000 lux(16 hour/day)throughout the period. Kinetin (0.01μM)and 2, 4-D(0.01μM)were added to the culture medium. The induced callus was subcultured in the dark at one month intervals on the same solid medium as that for callus induction.

Isolation of protoplasts

Protoplasts were prepared from the fully expanded young fruit and callus tissues induced from the flesh. The flesh was cut into small pieces, about 1 mm wide, with a surgical knife. About 0.2g(fresh weight) of flesh blocks or about 50mg(fresh weight) of callus were incubated in centrifuge tubes with 5ml of enzyme solution for 1 to 6 hours at 30℃ with shaking(90 reciprocation/min). For protoplast isolation, enzyme solution of Koyama *et al.*[2]was

modified and used in this experiment (Table 1). Cellulase "Onozuka" RS was used instead of Cellulase "Onozuka" R-10 which had been used by Koyama *et al.* Furthermore, 1% Meiselase P1 and Hemicellulase were added originally to the solution. The isolated protoplasts were washed three times with 0.6M D-mannitol solution by centrifugation at 100 xg for 5 min. The number of protoplasts was counted on a hemacytometer (Thoma counting chamber). Yields of protoplasts were determined by averaging triplicates in each treatment.

Table 1. Enzyme solution for isolating protoplasts.

Pectolyase Y-23	0.1%
Cellulase "Onozuka" RS	2.0%
Meiselase P1	1.0%
Driselase	0.5%
Hemicellulase	1.0%
D-Mannitol	0.6M
KH_2PO_4	1.0mM
NH_4NO_3	5.0mM
Sodium citrate	5.0mM
pH	5.8

This enzyme solution is modified from that of Koyama *et al.*

Culture and viability of protoplasts isolated from callus

Protoplasts were cultured with MS liquid medium containing 0.01μM of kinetin, 0.01μM of 2, 4-D and 0.6M D-mannitol without adding saccharose in micro plates (Corning Cell Wells) at 25℃ in the dark. The density of protoplasts was finally adjusted to 1.0 X 10^5 ml^{-1}. Protoplasts were observed using an Olympus phase contrast microscope BHS-323 (Olympus Co., Japan) during the culture.

Results

Induction and culture of callus

In preliminary experiments to induce callus from fruit, more than 50% of the explants were contaminated without peeling treatment. In contrast, callus was induced successfully with low contamination (below 1%) by the peeling treatment of fruit. The media containing kinetin and 2, 4-D effectively induced callus as shown in Fig.1. Callus growth in the medium was fairly active and the callus continued to grow over 3 months without browning.

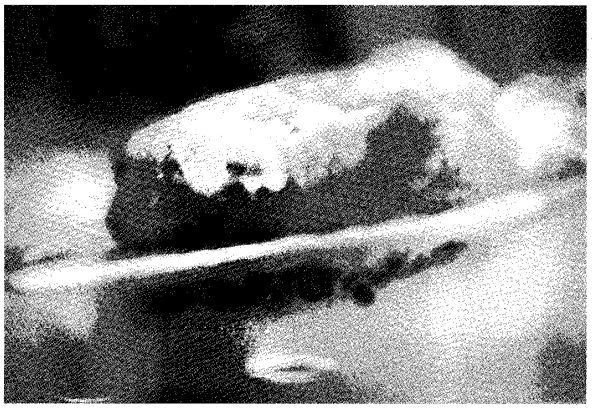

Fig.1 Callus formation from a peeled fruit of *G. baldianum*.

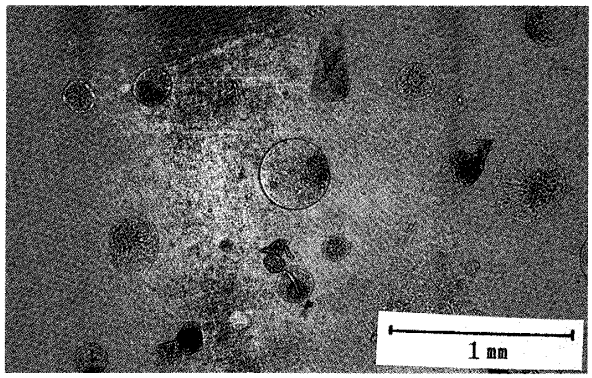

Fig.2 Fresh protoplasts isolated from callus.

Isolation of protoplasts

The diameter of protoplasts induced ranged from 250μm to 430μm. Protoplasts were isolated more easily from callus tissues (Fig.2).

Culture and viability of protoplasts isolated from callus

After protoplast culture for 24 hours, several masses composed of two cells showing first cell division were observed, and 4-cell-stage cells appeared after 2 days of culture. Then, many micro colonies were observed after 7 days of culture(Fig.3(a), (b)). Furthermore, colony formation was observed within 2 weeks(Fig.3(c)).

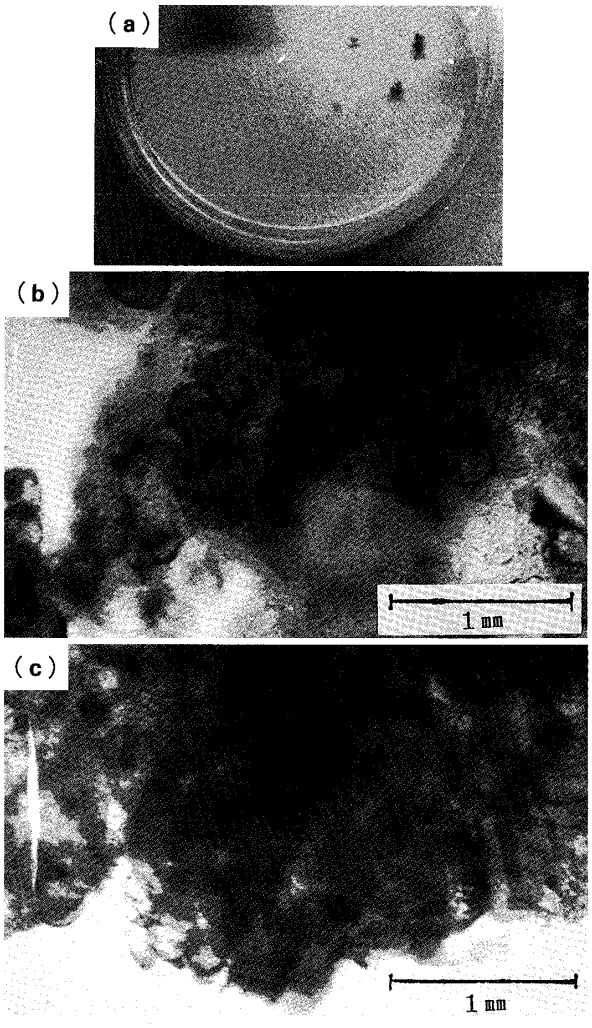

Fig.3 Colony formation.
(a)Colonies on the plate after 7 days culture.
(b)Colonies observed using microscope after 7 days culture.
(c)Colonies observed using microscope after 2 weeks culture.

Discussion

In general, sterilization of materials obtained from plants is difficult. This difficulty limits callus formation from plant of many species. In this experiment, after surface-sterization the flesh was peeled from fruit under aseptic conditions and then the flesh were inoculated in the callus induction medium. Consequently, callus could be induced with almost no contamination from the peeling treatment of fruit. This treatment resulted in shortening the sterilization time to only *ca.* 5 min sterilization time(70% ethanol for 30 sec and 1% sodium hypochlorite aqueous solution for 5 min). This sterilization time was satisfactory to prevent contamination. Callus was obtained successfully in the madia containing 0.01μM of kinetin and 0.01μM of 2, 4-D. These results suggest that the medium containing 0.01μM of kinetin and 0.01μM of 2, 4-D is suitable for the induction and culture of *G. baldianum* callus. In order to define the optimal condition, however, further research is needed to examine the effect of kinetin and 2, 4-D concentration.

Protoplasting is an important technique for somatic hybridization by cell fusion and for genetic improvement by gene transfer. In this study, determining the conditions of protoplast isolation and culture is the first step in transformation research of *G. baldianum*, although further investigations are still needed to establish the appropriate culture conditions for plantlet regeneration from protoplast derived callus.

Acknowledgments

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References

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2)Koyama, M., Hosoi, Y. and Saito, A., *J. Jpn. For. Soc.*, 70, 231-233(1988).