Isolation and Culture of Protoplast from Finger Millet (*Eleusine coracana*) Callus

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Abstract : High yield of protoplast was obtained from the green callus tissue of finger millet at one week after subculture on MS medium with 0.2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. The protoplasts were released using the enzyme solution containing 2% Cellulase YC and 0.2% Pectolyase Y-23. About 75% of the protoplasts cultured in the MS liquid medium with 0.4 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin supplemented with 5% coconut water were started to show regeneration of the cell wall within 12 h. Cell division was observed within 24 h after culture. The protoplasts that underwent division were about 15%. The sustained cell division could lead to microcolony formation in 4 d after culture. However, further growth did not occur and viability gradually decreased during culture.

Key words : Cell division, Cell wall, Finger millet, Protoplast isolation and culture

シコクビエ (*Eleusine coracana* Gaertn) カルスからのプロトプラスト分離と培養 : Totik Sri MARIANI・三宅 博・谷口 武 (名古屋大学農学部)

要 旨 : 0.2 mg l^{-1} 2,4-D と 0.1 mg l^{-1} カイネチンを含む MS 培地で 1 週間培養したシコクビエの緑色カルスから高収量のプロトプラストが分離できた。この分離には 2% セルラーゼ YC と 0.2% ペクトリアーゼ Y-23 を含む酵素液を用いた。プロトプラストを 0.4 mg l^{-1} 2,4-D, 0.1 mg l^{-1} カイネチン及び 5% ココナッツ液を含む MS 液体培地で培養すると 12 時間までに 75% のプロトプラストで細胞壁の形成が始まった。細胞分裂は培養後 24 時間以内に認められ、その割合は約 15% であった。培養 4 日後には分裂の継続した細胞では小型のコロニーを形成した。しかし、その後培養中にそれ以上の増殖はなく、また生存率は次第に減少した。

キーワード : 細胞分裂, 細胞壁, シコクビエ, プロトプラストの分離と培養。

Finger millet is a millet that was domesticated in North eastern tropical Africa. This C_4 plant¹³⁾ is one of the important crops in human foods as well as genetic resources¹⁵⁾.

Investigations on tissue culture of finger millet have been done extensively, for example, somatic embryogenesis^{5,6,8,16)}, the induction of enlarged apical dome and multi shoot formation¹⁸⁾, callus initiation and plant regeneration¹⁴⁾. However, study on protoplast isolation and its culture of finger millet has never been reported. Therefore, considering the successful and high plant differentiation from callus of finger millet, it was attempted to isolate and culture the protoplast from the regenerable callus of finger millet. In this experiment protoplast isolation was examined and the morphological changes which occurred during culture were also investigated.

Materials and Methods

Plant material Seeds of finger millet were used as the source material.

Callus induction The seeds of finger millet were surface sterilized with 70% alcohol for 30 s followed by 1% sodium hypochlorite

for 5 min and washed three times in sterile water. Then the sterilized seeds were sown on the callus initiation medium (Tabel 1) and subcultured every 2 weeks. After 2 months, the green callus was transferred to the callus maintenance medium and subcultured weekly.

Protoplast isolation and culture The calli were incubated in the several kinds of enzyme solution (Tabel 2) for 3 to 4 hr, then filtered through a double layer of Miracloth and centrifuged. After being washed in washing solution (containing 0.5 M mannitol and 10 mM CaCl_2) 3 times, the protoplast suspension was filtered once more through one layer of Nylon filter (350 mesh). After that the protoplasts were centrifuged and then suspended in the protoplast culture medium with density of 10^6 protoplast ml^{-1} (Tabel 1). The protoplast suspension was placed in the plastic petri dish and incubated in the dark at 25°C .

Fluorescence and light microscope Freshly isolated protoplasts and protoplasts (1 to 5 days, 1 and 2 weeks after culture) were observed by fluorescence and light micro-

scope. Cell wall development was determined by Calcofluor White staining⁹⁾ and the viability of protoplasts and cells were determined by the fluorescein diacetate method¹¹⁾. Cell division was observed both by light and fluorescence microscopy.

Results and Discussion

Callus initiation and maintenance

Callus formation in the initiation medium gave a similar result as described by Wakizuka *et al.*¹⁸⁾. The green knob-like callus appeared between two emerging leaves which were ac-

companied with white unorganized callus. When these calli were subcultured in the same medium, a white friable callus always appeared surrounding a compact green callus. Therefore it was difficult to maintain only the compact green callus. In order to overcome this problem, the green calli were transferred to the callus maintenance medium which contained the lower level of 2,4-D concentration. The compact and organized green calli retained the morphogenic potential for a long time period (more than 8 months). Morphogenic potentials were checked by transferring the

Table 1. Composition of callus initiation and maintenance media and protoplast culture medium.

Constituent	CIM (mg l ⁻¹)	CMM (mg l ⁻¹)	PCM (mg l ⁻¹)
Murashige & Skoog Basal Medium	+	+	+
Thiamine-HCl	1	1	1
Casamino acid	500	500	500
2,4-D	3	0.2	0.4
Kinetin	0.1	0.1	0.1
Coconut water	—	—	5%
Mannitol	—	—	0.5 M
Sucrose	2%	2%	0.059 M
CaCl ₂ · 2H ₂ O	—	—	10 mM

CIM=Callus initiation medium

CMM=Callus maintenance medium

PCM=Protoplast culture medium

Table 2. Composition of enzymes solution.

Constituent	Enzymes solution			
	A	B	C	D
Cellulase YC	2%	—	1%	—
Cellulase R-10	—	2.5%	—	—
Pectolyase Y-23	0.2%	—	—	—
Meicelase P-1	—	—	—	4%
Pectinase	—	0.75%	0.8%	—
Macerozyme R 10	—	—	—	1%
Mannitol	0.5 M	0.45 M	0.45 M	0.7 M
CaCl ₂ · 2H ₂ O	10 mM	—	—	—
NaH ₂ PO ₄ · 2H ₂ O	0.7 mM	—	—	—
Potassium dextran sulphate	—	—	—	1%
Sulphur	—	—	—	17.3%
MES	3 mM	3 mM	3 mM	—
pH	5.6	5.6	5.6	5.8

calli to regeneration medium¹⁸⁾ (MS basal media containing 1 mg l^{-1} thiamin-HCl and 0.5 mg l^{-1} kinetin). Multiple shoots were emerging in 1 to 2 weeks after transfer while plantlets were obtained about 2 weeks later in the same medium. After two and a half months acclimatization, the plant produced inflorescence and seeds.

Protoplast isolation

Four kinds of enzyme solutions (Tabel 2) were used to release protoplasts from the callus. It was observed that only A enzyme solution could release a high amount of protoplasts in a short time (3–4 hr) compared to the others (Tabel 3). Digestion with D⁷⁾ and B¹⁾ enzyme solutions gave comparatively low protoplast yield and none of the protoplasts released in the C enzyme¹⁾. Combination of Cellulase YC and Pectolyase Y-23 seemed suitable for releasing the protoplasts from the compact green calli.

The difference of callus age affected the yield of protoplasts released in the enzyme solution. The best source for protoplasts was provided from the callus 7 days after subculture, which produced the highest amount of protoplasts, $3 \times 10^6 \text{ ml}^{-1}$ (Fig. 1). Protoplasts isolated from the callus more than 7 days after subculture contained many vacuolated protoplasts which were floating in washing solution in the washing process.

Protoplast culture

Change in viability, cell wall formation and cell division in finger millet protoplast culture was shown in Figure 2. The viability of freshly isolated protoplasts was quite high, about 85% (Fig. 4). However, the viability was gradually decreased during one and three day culture and markedly decreased to 10% on 5 days after culture (Fig. 12). By this time, browning of the protoplasts or cells appeared and it seemed influenced the viability of the whole protoplast culture. Kikuta et al. reported that freshly prepared protoplast of potato (*Solanum tuberosum* L.) leaves showing over 80% viability subsequently decreased to 50% after 24 hr incubation. It was also noted that the decreased viability was the first indication of the failure of cell wall regeneration¹⁰⁾.

Cell wall formation on the protoplast surface started 4 hr after culture, judging from calcofluor white staining (Fig. 5). Within 12 hr after culture, the protoplasts regenerated

the cell wall on the entire surface of protoplast (Fig. 6). The amount of cell wall regenerating protoplasts increased until 3 days after culture. At this time, the protoplasts regenerating cell wall were about 75%. However, after 5 day culture no cell wall formation was observed.

Cell division showed a similar pattern with cell wall formation activity. One day after culture, about 7% of protoplasts divided. Divided cells increased to 15% on the 3 day culture and formed microcolonies on the 4th days after culture. However, no cell division was observed on the 5th days after culture, because the viability of the cultured protoplasts was very low.

In more detailed observation, the isolated protoplasts can be classified into 4 types (Fig. 3), namely :

- Type **a** : richly cytoplasmic protoplasts.
- Type **b** : chloroplast containing protoplast with very fragile plasma membrane.
- Type **c** : vacuolated protoplasts with the cytoplasm pushed against plasma membrane or even with no cytoplasm.
- Type **d** : spontaneous fusion body of protoplasts.

Each type of protoplast (type **a, b, c** and **d**) showed the different response when cultured in the liquid medium, as shown in Table 4.

The **a** type protoplasts could regenerate cell wall 4 hr after culture whereas the **b** and **d** type protoplasts formed the cell wall 12 hr after culture. The **c** type of protoplasts regenerated the cell wall later, 2 days after culture.

One day after culture, budding protoplasts were often found in the **a** and **c** type protoplast. However, the budding protoplasts were found more in the **c** type protoplast than **a**

Table 3. The effect of enzyme solutions on the protoplast yield from finger millet callus.

Enzyme solution	Protoplast yield
A	+++
B	+-
C	-
D	+

+++ = many, + = few, +- = very few.
- = none

Fig. 1. Effect of the age of finger millet callus on the yield of protoplasts.

type. When the budding protoplasts were stained with calcofluor white, only one part of the protoplasts formed a cell wall whereas another part failed in regenerating a cell wall (Fig. 9). Fluorescence diacetate staining in the budding protoplast visualized that the daughter cell has weaker fluorescence than the mother cell (Fig. 10). Burgess⁴) explained that the phenomenon of budding has itself been used as a criterion of wall formation. This is reasonable since the ability to produce an extrusion such as a bud clearly implies some mechanical rigidity at the surface of the parent protoplast. According to Burgess and Linstead³), budding is an example of a more less abnormal shape change during culture. It results from the formation of a wall which is too weak to restrain the expansion of the protoplast within it. Our results also indicate that an extrusion such as a bud may be produced due to cell wall weakness.

Dividing protoplasts were observed only in the **a** type of protoplasts which occurred on 1 day culture. When the divided protoplasts stained with calcofluor white, they showed the blue fluorescence. This means that the protoplasts regenerated the cell wall completely and then divided (Fig. 7). Two days after culture, one cell of **a** type divided into three cells (Fig. 8).

The microcolony was observed on the 4th days after culture. However the percentage of

the microcolony during culture was very low, about 5% (Fig. 11), indicating only the small fraction of **a** type protoplasts sustained division leading to the microcolony formation. **c** type protoplasts remained as single cell or budded whereas **b** and **d** type protoplasts burst within a longer period of culture. A similar result was described by Lu et al.¹²): that the vacuolated non-embryogenic protoplasts as well as the spontaneous fusion products isolated from embryogenic suspension culture of *Panicum maximum* did not divide in culture. Bhojwani and White²) reported that mesophyll protoplasts of white clover derived cells and colonies turned brown when multicellular colonies were transferred to light after 2 weeks of culture in the dark. Frequent microscopic observations of dark grown cultures were also detrimental to dividing cells and cell colonies, because of the light-induced browning of the cells. Moreover, higher plating densities or delay in diluting the osmoticum resulted in the appearance of a brown precipitate. In this experiment, browning precipitate was caused by the bursting of protoplasts in the early period of culture.

Viability determination of protoplast culture showed that **a** type protoplasts have moderate viability, while **b** and **d** type protoplasts have low viability because they burst easily during culture. In contrast to **c** type protoplast, their viability was high because they were still

Fig. 2. Changes in viability, cell wall formation and cell division in finger millet protoplast during culture.

Table 4. Response of protoplasts in the liquid culture medium

Type of protoplasts*	Regeneration of protoplasts				Viability
	Cell wall	budding	dividing	microcolony	
a	4 hr	+ - /1 day	1 day	4 day	moderate
b	12 hr	—	—	—	low
c	2 days	+ + /1 day	—	—	high
d	12 hr	—	—	—	low

+ + = many, + - = few, — = none.

a = richly cytoplasmic protoplasts.

b = chloroplasts containing protoplasts.

c = vacuolated protoplasts.

d = spontaneous fusion body of protoplasts.

viable in the 2 weeks after culture. However, no cell division was observed from c type protoplasts.

In this experiment, it was observed that the protoplasts could be easily isolated from the green and compact callus of finger millet in 3 to 4 hr. The protoplasts could regenerate the cell wall, divide and form microcolonies. However, the browning precipitate occurring in the culture seemed to disturb the growth of the protoplast, cell and microcolony. The type of protoplasts also affected the protoplast regeneration. The a type protoplasts (richly cytoplasmic protoplasts) seemed promising for further experiments. The result of the present

study revealed the difficulty of culturing cereal protoplasts which prove recalcitrant. According to Vasil¹⁷⁾, the culture of cereal and grass protoplasts has proved to be extremely difficult, and only protoplasts isolated from cell cultures have been shown to undergo sustained cell divisions. Therefore, the suitable protoplast culture and physical environment must be extensively investigated. Since up to now there is no report about the isolation and culture of protoplasts from finger millet callus, this result may be useful in advance.

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Fig. 3. Light micrograph of freshly isolated protoplast : A ($\times 417$), B ($\times 390$), C ($\times 425$). a = richly cytoplasmic protoplast ; b = chloroplast containing protoplast ; c = vacuolated protoplast ; d = spontaneous fusion body of protoplast.

Fig. 4. Fluorescence micrograph showing the viability of protoplast immediately after isolation stained with fluorescein diacetate ($\times 883$).

Figs. 5 and 6. Fluorescence micrographs illustrating regenerated cell wall of protoplast after culture stained with calcofluor white ; 4 hr, showing the spots of fluorescence of calcofluor white (Fig. 5) ($\times 850$) ; 12 hr, completely covered with the fluorescence of calcofluor white (Fig. 6) ($\times 778$).

Figs. 7 and 8. Fluorescence micrographs showing the cell division stained with calcofluor white ; 1 day, first division of cell (Fig. 7) ($\times 800$) ; 2 days, one cell divided into 3 cells (Fig. 8) ($\times 753$).

Figs. 9 and 10. Fluorescence micrographs visualizing the budding protoplasts ; 1 day, stained with calcofluor white (Fig. 9) ($\times 824$) ; 1 day, stained with fluorescein diacetate (Fig. 10) ($\times 775$).

Fig. 11. Light micrograph showing the microcolony 4 days after culture ($\times 387$).

Fig. 12. Fluorescence micrograph showing the viability of microcolony 5 days after culture, stained with fluorescein diacetate ($\times 714$).

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