Organogenesis in Leaf Protoplast-Derived Calli of Mungbean*

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Abstract : Protoplasts were successfully isolated from mungbean leaves (*Vigna radiata* L. Wilczek) using an enzyme solution containing 4% Meicelase P-1, 1% Macerozyme R-10, 1% potassium dextran sulphate and 0.5 M mannitol. Approximately 4.1×10^7 protoplasts per gram fresh weight were released after digestion with an enzyme for 4 hours in the dark on a shaker. The cultured protoplasts started to divide 5 days after culture followed by sustained division, and a microcolony was produced on day 15 of culture. Microcalli appeared on the medium surface a month after culture and were large enough for transfer to a callus induction medium after 1.5 month of culture. Having transferred to the regeneration media, green and compact calli were developed in MS medium supplemented with NAA and BA. Globular-like calli appeared in the MS basal medium or in the mixture of MS salts and B5 vitamin supplemented with 2, 4-D. Root formation then occurred from this calli.

Key words : callus, mungbean, organogenesis, protoplast, root formation.

リョクトウ葉由来プロトプラストより生成したカルスにおける器官形成 : Iriawati・三宅 博・谷口 武 (名古屋大学農学部)

要 旨 : リョクトウ (*Vigna radiata* L. Wilczek) 葉より 4% Meicelase P-1, 1% Macerozyme R-10, 1% デキストラン硫酸カリウム塩および 0.5 M マンニットを含む酵素液を用いてプロトプラストを効率的に分離した。暗条件で振とう培養し、酵素処理 4 時間後に新鮮重 g 当り約 4.1×10^7 個のプロトプラストが得られた。プロトプラストは培養後 5 日で分裂し始め、その分裂は継続し、培養 15 日でマイクロコロニーを形成した。培養 1 月後に培地表面にマイクロカルスが現れ、1.5 月後にカルス誘導培地に移すことのできる大きさになった。再分化培地に移したとき、NAA と BA を含む MS 培地では緑色のコンパクトカルスが生じた。2, 4-D を含む MS 基本培地または MS 無機塩と B5 ビタミンの混合培地では球状カルス生じた。このカルスから根の形成がおこった。

キーワード : カルス, 器官形成, 根形成, プロトプラスト, リョクトウ。

Recently attempts to regenerate the isolated protoplast from leguminous plants had been performed by several researchers^{1,7,10,11,17,19}. However, only a few of those plants indicated competence for producing a whole plant. In mungbean plants, the success in plant regeneration from tissue culture are still scarce^{5,8,12,13}. Furthermore, the information on protoplast isolation and culture is insufficient as well. Attempts to regenerate a plantlet from protoplasts of mungbean have been undertaken by some researchers. Nevertheless, plantlets have never been obtained. Root protoplasts can only proliferate in some calli¹⁹. Root and leaf-like structures were developed from leaf and hypocotyl protoplasts of this plant, however

no further growth has been observed⁴.

Present experiment was undertaken to establish a suitable medium for promoting plant regeneration from leaf protoplasts of mungbean.

Materials and methods

1. Plant material

Seeds of mungbean (*Vigna radiata* L. Wilczek var. Merak) were surface sterilized with 70% ethanol for 30 s, followed by 2% sodium hypochlorite for 15 min and were then rinsed several times with sterile distilled water. Seeds were sown on agar-solidified MS basal medium¹⁴ without a plant growth regulator and placed in a culture room at 25–27°C under 1000 lux of illumination.

2. Protoplasts isolation and purification

Primary leaves were excised from 7–8 day-old seedling and cut into fine pieces of approximately 0.5 mm². They were then digested with five different enzyme solutions containing Cellulase R-10, Cellulase YC

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Abbreviations : 2,4-D, 2,4-dichlorophenoxyacetic acid ; IAA, Indole-3-acetic acid ; NAA, α -naphthalene acetic acid ; BA, 6-benzyladenine ; KIN, kinetin.

Table 1. Composition of enzyme solutions for protoplast isolation.

Constituents	Enzyme solutions				
	A ⁴⁾	B	C	D	E
Cellulase R-10	1%	—	1%	—	1%
Cellulase YC	—	2%	—	—	—
Meicelase P-1	—	—	—	4%	—
Macerozyme R-10	0.2%	0.5%	1%	1%	1%
Mannitol	0.6M	0.5M	0.6M	0.5M	0.3M
CaCl ₂	—	—	5.0mM	—	2.5mM
Potassium dextran sulphate	—	—	—	1%	—
pH	5.8	5.7	5.8	5.8	5.8

Table 2. Composition of media for protoplast culture

Constituents	Medium		
	I	II	III
Macronutrient	MS ¹⁴⁾	KM8P ¹⁰⁾	KM8P
Micronutrient	V47 ²⁾	KM8P	KM8P
Vitamin & organic acids	V47	KM8P	KM8P
Sugar	V47	KM8P	KM8P-mod (mannitol 81.9 gr)
Coconut water	—	20ml	20ml
Hormones	NAA	NAA	NAA
	BA	2,4-D	2,4-D
		Zeatin	Zeatin

(Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) or Meicelase P-1 (Meiji Seika Co. Ltd., Tokyo, Japan), combined with Macerozyme R-10 (Yakult Biochemicals Co. Ltd., Nishinomiya, Japan) and mannitol, supplemented with potassium dextran sulphate or calcium chloride (Table 1). The pH of enzyme solutions were adjusted to 5.8 by addition of 0.1 N HCl. Incubation of enzyme-leaves mixture was carried out for 4 h on a shaker at room temperature in the dark. After enzyme treatment, protoplasts were then separated from undigested cellular material by passing through 350 μ m nylon-mesh followed by centrifugation at $100\times g$ for 5 min and washed once in washing solution which consisted of 0.5 M mannitol and 2.5 mM calcium chloride. Further purification of protoplasts was carried out by resuspending them in washing solution and centrifuging them twice at $100\times g$ for 2 minutes each.

3. Protoplasts culture

The purified protoplasts were resuspended in the different culture media (Table 2), liquid

or solidified with 1.2% agarose, at a final density of 10^5 – 10^6 protoplasts/ml. Approximately 14 protoplast-containing agarose droplets were allowed to solidify on the bottom of a small sterile plastic Petri dish (Falcon 50 $\times$ 9 mm) for 15 min at room temperature. Three milliliters of medium were then poured into the plastic dish submerging the droplets. For liquid culture, 3 ml of protoplast suspension in different medium mixture were poured into the plastic dish. For the first 2 weeks, protoplasts were cultured in the dark at room temperature. The medium was subsequently changed with a fresh and gradually reduced osmolarity-medium every week until the small visible calli appeared on the agarose surface.

4. Viability and cell wall formation tests of protoplasts

The viability of protoplasts was determined by fluorescein diacetate (FDA) staining³⁾. Cell wall formation of protoplasts was examined using Calcofluor White¹⁵⁾. Protoplasts were stained with 0.01% Calcofluor White for 5 min, followed by twice rinsing in washing

solution. Both viability and cell wall formation tests were investigated under a Nikon fluorescence microscope.

5. Callus induction and plant regeneration

When the small calli (1–2 mm in diameter) were developed they were transferred to the callus induction medium which consisted of MS basal medium¹⁴⁾ supplemented with 1.5 mg/l 2,4-D and 0.5 mg/l KIN until they were large enough for being transferred into the regeneration media. The culture were maintained in the light at 1200 lux at room temperature (25°C) with subculturing every 2 weeks. After one month in the callus induction medium, the calli were then transferred into regenerating media that consisted of MS basal media¹⁴⁾ supplemented with various concentrations of KIN or BA (0.2–10 mg/l), with or without 2, 4-D, IAA or NAA (0.2–2 mg/l) or in a medium which consisted of MS salts¹⁴⁾ in a mixture with B5 vitamin⁶⁾ supplemented with KIN (5 mg/l) and/or 2, 4-D (0.2–1 mg/l).

Results

A high yield of protoplasts, approximately 4.1×10^7 protoplasts per g fresh weight, was obtained from leaf tissues digested with the enzyme solution containing 4% Meicelase P-1, 1% Macerozyme R-10, 1% potassium dextran sulphate and 0.5 M mannitol (Fig. 1, D). The protoplasts were successfully purified by filtration through a nylon filter 350 μ m mesh and their diameter was relatively similar (20 μ m), with chloroplasts dispersed around the cytoplasm (Fig. 2). The other enzyme solution produced only a few protoplasts. The combination of Cellulase YC and Macerozyme could also produce a large amount of protoplasts, however the yield was lower than the Meicelase – Macerozyme combination. Therefore, the former mixture was then used for the isolation of mung bean leaf protoplasts.

The viability of the protoplasts was still high after 3 days of culture in both liquid and solidified media. It was proved by a strong fluorescence which appeared after FDA staining (Fig. 3). Cell wall formation was initiated 4 h after culture judging by small fluorescent spots of Calcofluor White on the protoplast surface. One day later, Calcofluor White fluorescence was observed surrounding the cell

Fig. 1. Effect of enzyme solutions on the yield of mungbean protoplasts. (A, B, C, D and E see : Table 1).

(Fig. 4.)

Protoplasts slowly grew in the liquid medium and had a strong tendency to form an aggregation. First the enlargement of protoplasts occurred, and subsequently divided after 9 days in culture (Fig. 5). However, they turned brown 14 days after culture. When protoplasts were cultured in agarose-solidified medium II, the first cell division took place 5 days after culture (Fig. 6). At day 8 of culture, double, triple and tetrad cells as a result of second and third division were observed in the culture medium (Figs. 7 and 8). Sustained division resulted in a microcolony (6–15 cells) which initially came out 15 days after culture and subsequently grew into a large colony 23 days after culture (Fig. 9). Small and greenish microcalli emerged on the surface of agarose a month after culture (Fig. 10). The calli reached 1–2 mm in diameter when they were transferred to the callus induction medium. The protoplasts could also grow in medium I and III. However the growth of the cluster cells was then ceased after 2 weeks in medium I, whereas in medium III, the protoplasts had reached a microcallus when browning appeared after 2 months in culture.

The pale green and compact calli were formed when the microcalli were cultured in callus induction medium containing KIN and 2,4-D. Having transferred to the regeneration medium, a soft and compact green calli were

Explanation of figures

- Fig. 2. The freshly isolated mungbean protoplasts. (Bar $50\ \mu\text{m}$)
- Fig. 3. Mungbean leaf protoplasts after being treated with fluorescein diacetate show a high viability after 24 h of culture. (Bar : $50\ \mu\text{m}$)
- Fig. 4. Mungbean leaf protoplasts after being treated with Calcofluor White show the cell wall surrounding the mungbean protoplasts one day after culture. (Bar : $50\ \mu\text{m}$)
- Fig. 5. The first division of mungbean leaf protoplasts 9 days after culture in the liquid medium. (Bar : $50\ \mu\text{m}$)
- Figs. 6-10. Microcalli formation from leaf protoplasts of mungbean. Fig. 6: The first division of protoplasts which occurred at day 5 of culture in semi-solid medium. Figs. 7 and 8: The second and third division, respectively, after 8 days in culture. Fig. 9: The large colony which was observed 23 days after culture. Fig. 10: Microcalli appear after a month in culture. (Bar : $50\ \mu\text{m}$)
- Fig. 11. Compact green calli arise after culture in MS medium supplemented with NAA and BA.
- Fig. 12. Globular-like calli (arrow), root initiation (arrow head) (A) and root formation (B) from protoplast-derived callus following culturing in the medium containing 2,4-D.

found in the medium containing BA and NAA. The calli grew well in the medium containing 1.5 ppm NAA and 5 ppm BA, or in the same medium supplemented with casein hydrolysate (Fig. 11). The similar appearance was noted in calli grown on a medium containing 0.5 ppm NAA and 5 ppm BA, however the calli seemed softer than the former. Calli regenerated from protoplasts were still undif-

ferentiated in these media. The globular-like calli developed following subculture in the MS basal medium containing 2, 4-D (1-2 mg/l) with 2% sucrose. In the mixture medium containing MS salts and B5 vitamins supplemented with 2, 4-D (1 mg/l) similar globular-like calli also appeared. These globular-like calli subsequently developed roots (Fig. 12), however shoots were not produced in the

present studies.

Discussion

A high yield of protoplasts could be easily released from mungbean leaf after digestion using Meicelase P-1 in combination with Macerozyme R-10. However, the combination of Cellulase R-10 and Macerozyme R-10, as previously described by Eapen⁴⁾, could not give a good result. The mixture of Meicelase-Macerozyme was also suitable for cell wall digestion and protoplast isolation from root tissue of mungbean when they were combined with hemicellulase¹⁹⁾.

The use of mungbean leaf as a source of protoplasts produced a good result in yield and uniformity compared to the other parts of this plant (data not shown). These mesophyll protoplasts were usually free from undigested cells and debris. Therefore the further purification by floatation using sucrose solution or sea water⁴⁾ was not necessary.

The age of explants influenced the yield of protoplasts. Maximum yields (4.1×10^7 protoplasts/g fresh weight) were obtained from young, fully expanded leaves from 7 day-old seedlings. The younger explants gave only a few protoplasts and the older ones produced protoplasts after a longer period of enzyme treatment and the yields were lower than the 7 day-old seedlings. The Kao and Michayluk medium⁹⁾, enriched with vitamins, organic acids, sugar, sugar alcohols, casamino acids and coconut water proved to be the best medium for callus regeneration from leaf protoplasts of mung bean. In addition, a high concentration of mannitol instead of glucose in medium III was insufficient for the growth of protoplasts. It seemed that mannitol played a role as an osmoticum only, whereas glucose had a role not only for osmoticum but for nutrition as well. Medium I, which had a composition that was not as rich as the medium II and III, indicated poor growth of protoplasts. Roest and Gilissen¹⁶⁾ noted that the basal medium and its supplements played an important role in the regeneration of protoplasts. It was also demonstrated that the medium KM8P was able to support cell wall formation and indefinitely division in *Vicia hajastana*⁹⁾.

The culture medium solidified with 1.2% agarose promoted the growth of protoplasts

compared to the liquid medium. The use of agarose was proved to be useful for avoiding the agglutination of protoplasts and to be beneficial for division and colony formation.

Protoplasts from a majority of the grain legumes were still difficult to induce for plant regeneration and only a small amount information was available from these plants. This difficulty also occurred in mungbean. At this moment, attempts to regenerate a plantlet from protoplasts of any part of this plant always meet with failure^{4,19)}. In the present study, leaf protoplast-derived calli only proceeded root formation after being cultured in the medium containing 2, 4-D.

Although similar procedures, enzyme solution and culture medium as described by previous workers, were conducted in the present experiment, they did not give similar results. In our experiment, the leaf protoplasts could grow well in medium KM8P and produced a microcalli after 6 weeks in culture and resulted in the globular-like calli and root formation on the tip of these calli. Attempts to induce shoot formation, moreover, in plant regeneration are still in progress.

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