SOYBEAN PROTOPLAST ISOLATION AND CULTURE*)
(Pemisahan dan Pembiakan Protoplas Kedelai)

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Intisari

Protoplas kedelai dipisahkan dari kotiledon muda (15 – 20 hari bunga mekar) yang direndam selama 5 jam di dalam larutan ensim dengan kandungan selulose 2%. Pembelahan sel teramati selang 3 hari sesudah pembiakan di dalam media cair KP8, apabila protoplas diikat dalam alginat berdaya lekat lemah dengan kadar 1%. Varietas Galunggung nampak lebih unggul dibandingkan dengan varietas lainnya dalam hasil protoplas dan kemampuan membelah.

Abstract

Soybean protoplasts were isolated from immature cotyledons (15 – 20 days after anthesis) incubated for 5 hours in the enzyme solutions containing 2% cellulase.

Cell division was observed after 3 days incubation in the KP8 liquid medium when the protoplasts were embedded in 1% low viscosity of alginate. Galunggung voriety was superior both in the protoplast yield and plating efficiency.

Abbreviation

2,4-D : 2,4-dichlorophenoxyacetic acid; BAP: 6-benzylaminopurine; MES: 2-N-morpholino ethane sulfonic acid.

Introduction

Plant protoplasts generally refer to all of the components of a plant cell excluding the cell wall. Therefore, protoplasts are useful tools for genetic manipulation (Mantell et al., 1985) and plant improvement. First report of the plant protoplast isolation is in 1960, when Cooking was able to isolate protoplast mechanically from tomato root tips. Afterward, *a lot* of efforts have been done and Takebe et al. (1971) were the first group that reported the protoplast regeneration in tobacco.

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In soybean, first report of plant regeneration from protoplasts was in 1988 by Wei and Xu. Protoplasts were isolated from immature cotyledons and cultured in the KP 8 liquid medium (Kao, 1977). Unfortunately, the frequency of regeneration was very low. Using similar explant and medium, Dhir et al. (1991) also reported plantlets recovery. The use of variety Clark-63 gave rise to high regeneration. Dhir et al. (1992) reported that from 14 genotypes, they were able to isolate the protoplasts. Howeyer, different plating efficiency and regeneration were observed. In this experiment, we try to assess the capacity of several Indonesian soybeans for protoplasts isolation and culture.

Materials and Methods

Soybean seeds from six varieties (Galunggung, Lompobatang, Rinjani, Tidar, Tainung and Wilis) which were obtained from the field station of the Secondary Crop Research Centre of Gunung Kidul, Yogyakarta, were grown in the greenhouse of Institute of Molecular Biology, Vrije Universiteit Brussel-Belgium both in pots and directly on soil. The culture conditions were 12 hours daylight and a temperature of 26°C during winter season. Pods (45 – 55 mm long) containing immature seeds of approximately 4 mm long (15 – 20 days after anthesis) were harvested, then kept at 4°C for 2 days. Pods were sterilized by immersing them 20 minutes in bleach water, rinsed 3 – 4 times in sterile distilled water. Immature seeds were excised from sterile pods. From them, immature embryos were isolated and cut into pieces.

Three enzyme mixtures (MSC1, MSC2 and MS6M) were exploited for protoplast isolation. The concentrations of CaCl2. 2H2O (5 mM), MES (5mM), macerozyme (0.5% w/L), and pectolyase (0.1% w/L) were similar in all of the three enzyme mixture. However, MSC1 and MS6M contained 2% cellulase and either 0.4 M or 0.6 M mannitol, while MSC2 contained 0.2% cellulase and 0.4 M mannitol. Enzyme solutions were filter sterilized (0.2 mm pore size of nalgene filter unit) just before used.

Immature embryo pieces were directly incubated in the enzyme solution in the dark either with or without agitation. Various incubation periods were used to assess their effect on the protoplast yield.

Released protoplasts were washed with W5 solution, filtered through 50 μm sieves and pelleted by centrifuation at 500 rpm for 5 minutes. Pelleted protoplasts were resuspended in 20% ficoll-400 solution in 0.4 M sorbitol and centrifuged again at the

same speed for 15 minutes. The floating protoplast band was taken up, further washed with 0.5 M sorbitol, resuspended and centrifuged at the same speed for 5 minutes. The pellet was harvested and diluted in the sorbitol solution and the number of protoplasts per ml was estimated. Protoplasts were adjusted to a density $1 - 2 \times 10^5$ each ml.

Protoplasts were diluted in the ratio 1 : 1 with 2% low viscosity alginate in 0.4 M sorbitol solution and mixed gently. They were incubated for 1 – 2 hours and 0.5 ml of the protoplasts was taken up and poured onto a solid medium containing 20 mM CaCl2, 0.4 M sorbitol and 1% agar (Ca-agar solid medium) at 3.5 cm petri dishes for polymerization. After overnight incubation, the alginate discs were removed carefully and transferred to 6 cm petri dishes containing 5 ml KP8 liquid medium. Petri dishes were sealed with parafilm and cultured in the dark at 26°C. The cultures were checked daily and liquid medium was replaced every 10 days.

While microcolonies developed, the cultures were transferred to light condition. Plating efficiency (ratio number of divided protoplasts to total protoplasts culture) was determined and medium was replaced by a liquid embryo induction medium composed of MS salt (Murashige and Skoog, 1962), B5 vitamin (Gamborg et al., 1968), 0.5 mg/L 2,4-D, BAP and kinetin respectively. Green microcalli (2 – 3 mm in size) were then incubated on solid medium for embryo induction.

Results and Discussion

1. Capacity of defined enzyme solutions for protoplasts isolation

Table 1 showed that immature cotyledons gave positive reaction in the enzyme solution contained 2% cellulase (MSC1 and MS6M) even without agitation. In this enzyme solution, a lot of free protoplasts was already observed after 5 hours incubation. A longer incubation increased the number of liberated protoplasts, unfortunately they were more fragile. To obtain similar result, a longer period of incubation (overnight) was required by the enzyme solution containing a low percentage of cellulase (0.2%). When gentle agitation was introduced, effect was observed. For all of the enzyme solutions, 5 hours incubation with agitation has caused the occurance of cell fusion from liberated free protoplasts. Longer incubation (up to 7 hours) increased the number of fused cells. Overnight incubation resulted in the breakdown of the liberated free protoplasts in the enzyme solution.

Table 1. **Capacity of defined enzyme solutions for immature cotyledon protoplast isolation.**

enzyme solution	incubation time (hour)	agitation	
		with	without
mscl	5	+	+
	6	+	+
	7	+	+
	overnight	+	+
msc2	5	+	−
	6	+	−
	7	+	−
	overnight	+	+
ms6m	5	+	+
	6	+	+
	7	+	+
	overnight	+	+

remarks:
+) there is enough amount of protoplast to be isolated.
−) there is not enough amount of protoplas to be isolated.

Result from this experiment led to recommendation that immature cotyledons should be incubated in the MSC1 or MS6M enzyme solution for more than 5 hours in order to obtain a sufficient amount of good soybean protoplasts.

2. Protoplast yield and plating efficiency

Table 2 showed the protoplast yield per gram fresh weight of immature cotyledons incubated for 5 hours without agitation. It was clear that Galunggung variety appeared to be the best. When the protoplasts were embedded in alginate, then cultured in the KP8 liquid medium, a 3 days incubation was enough for the protoplasts to form a new cell wall and initiate division. With naked eyes, small microcolony was observed only after two weeks. Table 3 showed that Galunggung variety had the highest plating efficiency (18.64%) followed by Tainung, Lompobatang and Rinjani. Protoplasts isolated from Tidar and Wilis were not developed during incubation in the KP8 medium. The cells looked brown in colour and did not divide. Dhir et al. (1992) reported that protoplast yield and plating efficiency were genotype dependent. Different plating efficiency sometime was caused by specific sugar and salt requirements in the early stages of development. Using different genotypes, higher

Table 2. **Protoplast yield isolated from one gram fresh weight of immature soybean cotyledons.**

variety	number of protoplast/ g explant (10^6)
Galunggung	21.60
Lompobatang	8.01
Rinjani	13.69
Tidar	7.98
Tainung	3.80
Wilis	3.04

Table 3. **Plating efficiency of protoplast isolated from immature soybean cotyledon.**

variety	plating efficiency
Galunggung	18.64
Lompobatang	2.97
Rinjani	1.77
Tidar	ND
Tainung	4.67
Wilis	ND

remarks:
ND: not detected.

plating efficiency was reported by Wei and Xu (1988) and Dhir et al. (1992).

When the cultures were transferred to the light condition, some of the small microcolonies were becoming green in colour. Green microcalli were picked up and sub-cultured into solid medium for regeneration. After 3 subsequent sub-cultures of 14 days each, these calli grew to 5 – 10 mm in size and became bright green in colour and compact in shape. Nodular form was not observed, although roots were found. Plantlets recovery was not yet successfull.

Conclusion and Future Perspectives

1. Enzyme solution contained 2% cellulase was neccesary to produce enough amount of protoplast.
2. Five hours incubation without agitation was required to have good protoplast quality.

3. Galunggung variety was superior both in protoplast yield and plating efficiency.
4. Rooting from calli derived protoplast was obtained.

The effort to regenerate plantlet from protoplast have not been succeeded yet, hovewer, rooting calli was observed. Probably, some treatments in order to induce shoot formation must be evaluated, for instance dessication of microcalli, the use of abscisic acid in which in general induce the formation of embryo from calli derived protoplast.

As soybean protoplast could be isolated and cultured, gene transfer experiment by making use of protoplast such as PEG (polyethelene glycol) mediated gene transfer or electroporation system can be carried out.

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