ORIGINAL ARTICLE

Isolation and culture of protoplast from leaves of *Lactuca sativa*

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Abstract

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Protoplasts were isolated from leaves of lettuce (*Lactuca sativa* L.) seedlings after *in vitro* germination for 25, 30, 40 and 50 days. The leaves were stripped and incubated in various combinations of cellulase and pectinase. Protoplasts were cultured on MS medium containing various kinds and concentrations of plant growth regulators in different culture systems including liquid media, hanging, drop culture and solid media. Results revealed that the highest number of viable protoplasts, $14.1x10^5$ cells per gram of fresh weight, was obtained from 30 day-old leaves of lettuce seedlings and isolated by using 2% cellulase in combination with 1% pectinase. Liquid MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA promoted the highest cell division up to 17.67%. First division of protoplasts was observed at 4 days after culture and microcolony formation occurred at the 4th week after culturing. Unfortunately, neither callus formation nor plantlet regeneration were obtained.

Key words : protoplasts, lettuce, isolation, microcolony formation

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บทคัดย่อ

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การแยกและเลี้ยงโพรโทพลาสต์จากใบผักกาดหอม

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การแยกโพรโทพลาสต์ของใบผักกาดหอม (*Lactuca sativa* L.) โดยใช้ใบผักกาดหอมที่เพาะจากเมล็ดในสภาพปลอดเชื้อที่มีอายุ 25 30 40 และ 50 วัน ด้วยเอนไซม์เซลลูเลสร่วมกับเพคติเนสความเข้มข้นต่าง ๆ โพรโทพลาสต์ที่แยกได้ทำการเพาะเลี้ยงด้วยอาหารสูตรมูราชิเกะและสกุค (Murashige and skoog) เติมสารควบคุมการเจริญเติบโตหลายชนิดที่มีความเข้มข้นต่างกัน ทำการเลี้ยงในอาหารเหลว แบบแขวน แบบหยด และเลี้ยงบนอาหารแข็ง จากการศึกษาพบว่าใบที่มีอายุ 30 วัน ที่ระดับความเข้มข้นของเอนไซม์เซลลูเลส 2% ร่วมกับเพคติเนส 1% ให้จำนวนโพรโทพลาสต์ที่มีชีวิตสูงที่สุด 14.1 x 10^5 โพรโทพลาสต์ต่อกรัมน้ำหนักสด การเลี้ยงโพรโทพลาสต์ด้วยอาหารเหลวเติมสารควบคุมการเจริญเติบโต NAA 0.5 มก./ลิตร ร่วมด้วย BA 0.5 มก/ลิตร ส่งเสริมให้มีการแบ่งเซลล์ได้มากที่สุด 17.67% การแบ่งเซลล์ครั้งแรกปรากฏให้เห็นหลังเพาะเลี้ยง 4 วัน และมีการสร้างไมโครโคโลนี (microcolony) หลังเพาะเลี้ยง 4 สัปดาห์ อย่างไรก็ตาม โพรโทพลาสต์ยังไม่สามารถพัฒนาไปเป็นแคลลัสและพืชต้นใหม่จากไมโครโคโลนีได้

ภาควิชาวิทยาศาสตร์ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยสงขลานครินทร์ วิทยาเขตปัตตานี อำเภอเมือง จังหวัดปัตตานี 94000

Lettuce (*Lactuca sativa* L.), known as a staple crop, is the most economically important salad crop in many countries. It is in the same class as potato, tomato, cabbage, onion and bean. In Thailand, the tropical weather is more suitable to grow leaf lettuce than head lettuce. Additionally, downy mildew, a fungal pathogen caused by *Bremia lactucae*, is found to be a major problem of lettuce. Only wild lettuce could resist this disease. (Beharav *et al.*, 2006; Chaimonkol, 2007). Lettuce can be regenerated from various different parts of its plant such as axillary bud and apical buds (Nishio *et al.*, 1987) cotyledons and hypocotyls (Webb *et al.*,1994). It can also be regenerated from protoplast which are amenable to suspension culture (Matsumoto, 1990). Plant regenerated from protoplasts has proved a very useful technique for crop genetic improvement and somatic hybridization. There have been some reports about protoplast culture of lettuce (Brow *et al.*, 1987). However, no research on protoplast culture of *L. sativa* has been reported in Thailand. One important aim in the genetic improvement of the *L. sativa* is resistance to *Bremia lactucae*, a major fungal pathogen of lettuce (Crute and Davis, 1977) and suitability to grow this plant in the southern region of Thailand.

This paper describes an effective method to isolate and culture protoplasts from leaves of *L. sativa*, from which the microcolonies were formed. This result could be a good reference for future research in regeneration of this plant from protoplast.

Materials and Methods

Plant materials and growth conditions

Seeds of *Lactuca sativa* L., purchased from Know You Seed Co, Ltd. were germinated *in vitro* on Murashige and Skoog (1962) (MS) basal medium and solidified with 0.7% agar. They were illuminated (2000 Lux) at 25±2°C for 14 h/day. Leaves at the date of 25, 30, 40 and 50 days after germination were used for protoplast isolation.

Protoplast isolation

An *in vitro* leaves of 25, 30, 40 and 50 days-old from *vitro* plants (10 g fresh weight, Fw)

were sliced into about 1 mm ribbons. The treated leaves were immersed into 10 ml of enzyme solution in 60 mm (diameter) Petri dishes. The enzyme solution consisted of 2 % cellulase and 1 % pectinase. In addition, the leaves of 30 days-old plants were macerated with various mixed of enzymes cellulase (2, 1.5 and 1%) and pectinase (1, 0.5 and 0.3%). The enzymes were dissolved in 0.7 M mannitol, 1.7 mM $MgSO_4.7H_2O$ and 2.99 mM $CaCl_2.2H_2O$ (pH 5.6). The incubation plates were sealed with Parafilm and placed at 25±2°C on gyratory shaker at 60 rpm in the dark for 5 h. Then protoplasts were filtered through 70 μm nylon mesh and washed twice with washing solution (0.7 M mannitol, 1.7 mM $MgSO_4.7H_2O$ and 2.99 mM $CaCl_2.2H_2O$) by centrifugation at 80 x g for 3 min. The protoplasts were then purified by floating on a solution of 21% (w/v) sucrose and centrifuged at 100 x g for 5 min, and then washed once with the washing medium. Protoplast were counted using a haemacytometer. The viability of protoplasts was examined by staining with 0.01% (w/v) Fluorescein diacetate (FDA) dissolved in culture medium. After 10-15 min they were showed under a fluorescense microscope. Viability was scored as the percentage of fluorescent protoplasts in comparison with total number of protoplasts.

Effect of phytohormones and culture method

Protoplasts were cultured at final density of $2x10^5$ /ml in MS medium supplemented with 0.1, 0.2 and 0.5 mg/l of 2, 4-D or NAA and 0.5, 1.0 and 3.0 mg/l of BA. The mixtures were osmotically adjusted with 0.7 M mannitol. Using a liquid thin layer culture, 3 ml of protoplast suspension was pipetted into 60 mm (diameter) plates. The plates were sealed with Parafilm and allowed to culture in darkness at 27±2°C. After 7 days, the culture was added with fresh liquid medium reducing mannitol into 0.5 M to promote further the cell division.

In another experiment, protoplasts at the density of $2x10^5$ /ml were cultured by 4 different methods, including of liquid thin layer culture, hanging drop, drop culture and liquid on agar culture. Efficiency of cell division and plating were calculated on the basis of the percentage of protoplasts that were transformed into microcolonies.

Experimental design and statistical analysis

The yields of protoplast were counted by haemacytometer and the average number of protoplasts per gram fresh weight were calculated. This experiment was conducted in a Completely Randomized Design (CRD) with ten replications. Duncan's Multiple Range Test (DMRT) was used for the statistical evaluation of experimental data.

Results

Protoplast isolation

The highest yield of protoplasts was obtained from 30 day-old leaves of *L. sativa* in 2% cellulase and 1% pectinase was $14.1x10^5$ protoplasts/gFw. The age of leaves more than 30 days was not suitable for protoplast isolation due to yield and viability of these protoplast were very low. The production of protoplasts from 50 day-old leaves gave the lowest yield of protoplasts ($8.10x10^5$ protoplasts/gFw) and the viability of these protoplasts was 77.77% (Table 1). The 30 day-old leaves were used for determination of the optimum enzyme mixed. Among six enzyme combinations tested (Table 2), 2% cellulase mixed with 1% pectinase was the optimum enzyme mixed for protoplast isolation with the viability of 82.75% and the highest protoplast yield. Protoplast isolation in 1% cellulase mixed with 1% pectinase gave the lowest protoplast yield and viability ($2.2x10^5$ protoplasts/gFw and 79.17%, respectively). Increasing pectinase did not help to increase protoplast yield in this study. The protoplast contained uniformly distributed chloroplasts (Figure 1).

Effect of phytohormones and culture methods

Protoplasts were cultured in liquid MS medium supplemented with various concentrations of 2,4-D or NAA and BA. The result showed that NAA at the concentration of 0.5 mg/l in combination with 0.5 mg/l BA gave the highest cell divisions and development of protoplasts after culture for 4 and 7 days was 17.67% (+++). NAA

Table 1. Influence of different leave ages on protoplast isolation of *Lactuca sativa*. The enzyme solution consisted of 2% cellulase and 1% pactinase

Leave age (days)	Protoplast yield x 10^5 (cell/g.Fw.)	Viability of protoplast (%)
25	13.9 a	81.49
30	14.1 a	82.75
40	8.2 b	80.00
50	8.1 b	77.77 ns

Values (Mean) followed by the different letters within column are significantly different by Duncan's multiple range test ($p \leq 0.01$).

Table 2. Influence of enzyme combinations on the yield and viability of *Lactuca sativa* protoplasts

Combination of enzyme (%)		Protoplast yield (x10^5 cell/g.Fw.)	Viability of protoplasts (%)
Cellulase	Pectinase		
1	0.5	2.7 c	78.57
1	1	2.2 c	79.17
1.5	0.3	13.1 a	82.14
2	0.5	8.9 b	80.00
2	1	14.1 a	82.75

Values (Mean) followed by the different letters within column are significantly different by Duncan's multiple range test ($p \leq 0.01$).

promoted cell division better than 2,4-D. To observe the effect of BA on cell division, protoplasts did not show division in medium containing more than 1 mg/l BA. With 0.5 mg/l 2,4-D and 3.0 mg/l BA, cell division after culture in 4 days was 3.28% (+). The optimum combination of plant growth regulators for microcolony formation were found to be 0.2 and 0.5 mg/l NAA and 3.0 mg/l BA. However, 0.5 mg/l NAA gave the highest micro-colony formation (9.75%) (Table 3). The first cell division occurred after 4 days of culture. (Figure 2) and the percentages of cell division were 17.67% in liquid MS medium after culture for 4 and 7 days. Cell division in liquid on agar culture was 9.75% and microcolonies could be observed after 4 weeks while hanging drop and drop culture method were not suitable for cell division (Figure 3). Types of cultures were also tested to optimize cell divisions of the protoplasts. It was found that liquid thin layer culture method promoted the highest cell divisions of protoplasts (Table 4).

Discussion

Maximum yields and viability of *L. sativa* protoplast were obtained using 30 day-old leaves. This result is consistent the study with Webb *et al.* (1994) who reported that young leaves from 4-8 weeks-old plant were suitable for protoplast isolation. However, based on these observation, age of leaves effected for protoplast isolation due to the very old leaves had the complex composition such as the cell initiated of hemicellulose pectin lignin suberin and cutin. Among the enzymes used in this experiment, cellulase and pectinase were found to be effective for isolation of protoplasts

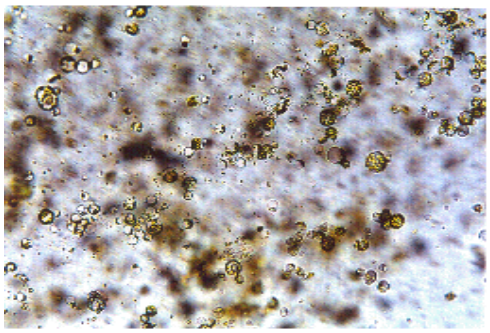

Figure 1. Fresh protoplasts isolated from leaf of *Lactuca sativa* with 30 day-old leaves and 2% cellulase and 1% pactinase.

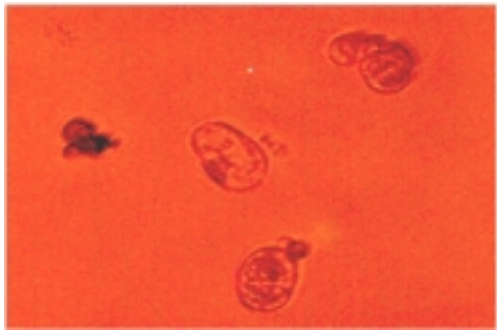

Figure 2. First cell division of *Lactuca sativa* protoplast in liquid MS medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA, 4 days after culture.

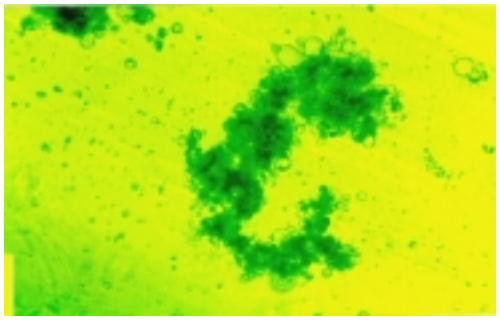

Figure 3. Microcolony formation in thin layer liquid MS medium of *Lactuca sativa* supplemented with 0.5 mg/l NAA and 0.5 mg/l BA 4 weeks after culture.
(Color figure can be viewed in the electronic version)

from the leaves. Engler and Grogan (1983) also reported that cellulase digested cell wall better than driselase hemicellulase and pectinase separated cells better than pectolyase y-23. To some plants its difficult to isolate protoplast using pectolyase y-23 since pectolyase y-23 consists of pectinlyase and polygalacturonase which is a catalyst for digest middle lamella. However, the optimum concentration of the cellulase was 1.0-3.0 and pectinase were 1.0-2.5% (Roberta, 1992).

It has been reported that a combination of enzymes was effective in protoplast isolation (Techato *et al.*, 2005; Sun *et al.*, 2005). Different combinations of plant growth regulator s for protoplast culture and colony formation were tested at various concentrations and the results are summarized in Table 3. It showed that the protoplast culture was achieved by using high concentration of auxin combined with low concentration of cytokinin. The optimum combination of plant growth regulators for initial cell division and microcolony formation was found to be 0.5 mg/l NAA and 0.5 mg/l BA. This result is consistent with the literature which reported that the combination of auxin and cytokinin was relatively effective for protoplast culture of *Lactuca saligna* (Brown *et al.*, 1987). Crut and Davis (1977) reported that addition of auxin NAA resulted in an increased cell division compared with 2,4-D and IAA. Cytokinin BA, kinetin and 2-pi were also important for cell division. The response of cultured protoplasts was to a large extent dependent on the culture system applied. The liquid culture method and liquid on agar culture improved the protoplast

Table 3. Effect of liquid MS medium containing combination of phytohormones on growth of *Lactuca sativa* protoplasts cultured at density of $2x10^5$/ml.

Phytohormone (mg/l)			Division of protoplast		Microcolony
2,4-D	NAA	BA	after 4 days	after 7 days	after 4 weeks
0.1		0.5	++	++	0
0.1		1.0	++	++	0
0.1		3.0	+	+	0
0.2		0.5	++	++	+
0.2		1.0	+	+	+
0.2		3.0	+	+	+
0.5		0.5	+	+	0
0.5		1.0	++	++	+
0.5		3.0	+	0	0
0.1		0.5	+	+	+
0.1		1.0	++	+	+
0.1		3.0	+	+	0
0.2		0.5	++	++	++
0.2		1.0	++	++	0
0.2		3.0	++	+	0
0.5		0.5	+++	+++	++
0.5		1.0	++	++	++
0.5		3.0	+	+	0

(0) no cell division, (+) cell division 3.28%, (++) cell division 9.75%, (+++) cell division 17.67%

Table 4. Effect of culture methods on development of *Lactuca sativa* protoplasts in MS medium supplemented with 0.5 mg/l. NAA 0.5 mg/l. BA.

Culture method	Division of protoplast		Microcolony
	after 4 days	after 7 days	after 4 weeks
Liquid thin layer	+++	+++	+++
Hanging drop	++	+	0
Drop culture	++	+	0
Liquid on agar culture	++	++	+

(0) no cell division, (+) cell division 3.28 %, (++) cell division 9.75 %, (+++) cell division 17.67 %

response. It has been reported that protoplasts could be more dispersed in the liquid culture than in the drop and hanging drop cultures. This may be due to protoplasts creating phenolic compounds that could be easily toxic to the surrounding protoplasts, therefore, both drop cultures were suitable only for low density protoplasts or protoclones (Nishio *et al.*, 1987). The positive effect which method has been shown in many kinds of plants (Karim and Adachi, 1997; Kuchuk *et al.*,

1998). To The author's knowledge, the micro-colony formation from the isolated protoplasts of *L. sativa* is reported in Thailand for the first time.

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