SHORT COMMUNICATION

Isolation and culture of suspension protoplasts of vetiver

Somporn Prasertsongskun

Abstract

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In this research, protoplasts were isolated from cell suspension derived from inflorescence of vetiver (*Vetiveria zizanioides* Nash) Surat Thani germplasm. The optimum condition for protoplast isolation was established by using 2% cellulase Onozuka R10, 2% macerozyme R10, 0.5% pectinase in 0.4 M mannitol and 7 mM $CaCl_2.2H_2O$ at pH 5.8 and incubated for 10 hours in the dark on the rotary shaker at 50 rpm. Maximum protoplast yields were 8.4×10^4 protoplasts/ml PCV. Division of protoplasts was observed only in liquid medium. The first cell division was observed after 3 days of culture initiation, and the average division was 5.0% in the N_6 medium supplemented with 1.0 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.5 mg/l BA (Benzyladenine). An optimal density for culture division was 1×10^5 protoplasts/ml.

Key words : protoplasts, vetiver, isolation, culture

Ph.D. (Bioscience), Asst. Prof., Department of Science, Faculty of Science and Technology, Prince of Songkla University, Muang, Pattani 94000, Thailand.
Corresponding e-mail: psomporn@bunga.pn.psu.ac.th
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บทคัดย่อ

สมพร ประเสริฐส่งสกุล

การแยกและเลี้ยงโพรโทพลาสต์จากเซลล์แขวนลอยของหญ้าแฝก

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จากการทดลองแยกโพรโทพลาสต์จากเซลล์แขวนลอยที่ได้จากการเพาะเลี้ยงช่อดอกของหญ้าแฝก (*Vetiveria zizanioides* Nash) แหล่งพันธุกรรมสุราษฎ์ธานี สภาวะที่เหมาะสมสำหรับแยกโพรโทพลาสต์ คือ ใช้เซลลูเลสอาร์ 10 ความเข้มข้น 2 % มาเซอโรไซม์อาร์ 10 ความเข้มข้น 2 % และเพกติเนสความเข้มข้น 0.5 % ในสารละลายแมนนิทอล 0.4 โมลาร์ และแคลเซียมคลอไรด์ 7 มิลลิโมลาร์ ที่ pH 5.8 บนเครื่องเขย่าความเร็ว 50 รอบ/นาที เป็นเวลา 10 ชั่วโมงในที่มืด โดยให้จำนวนโพรโทพลาสต์สูงที่สุด 8.4×10^4 โพรโทพลาสต์/มล. พีซีวี เมื่อนำโพรโทพลาสต์มาเลี้ยงจะมีการแบ่งเซลล์เฉพาะในอาหารเหลว พบการแบ่งเซลล์ครั้งแรกหลังจากเลี้ยงในอาหารสูตร N_6 เติม 2,4-ไดคลอโรฟีนอกซีอะซีติคแอซิด (2,4-D) ความเข้มข้น 1.0 มก./ล. และเบนซิลอะดีนีน (BA) ความเข้มข้น 0.5 มก./ล. เป็นเวลา 3 วัน ส่งเสริมการเกิดการแบ่งเซลล์ดีที่สุด (5.0 %) ความหนาแน่นที่เหมาะสมต่อการเลี้ยง คือ 1×10^5 โพรโทพลาสต์/มล.

ภาควิชาวิทยาศาสตร์ คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยสงขลานครินทร์ อำเภอเมือง จังหวัดปัตตานี 94000

Vetiver (*Vetiveria zizanioides* Nash) is an important grass. It is commonly called the "lowland" vetiver (Faek Hom in Thai). There are many populars germplasm of Faek Hom as following; Kamphaeng phet 2, Surat Thani, Songkhla 1, Songkhla 3 and Chiang Rai. Surat Thani germplasm has been found the most adaptive to general growing conditions in Thailand (Sayamanonta *et al*., 1996). It is a source of animal feed, thatching, handicrafts as well as soil erosion control. Vetiver has no rhizomes or stolons and is propagated by slips. However, there are reports of successful propagation of vetiver by tissue culture technique using leaves (Mucciarelli *et al*., 1993). Moreover, plantlets of vetiver can be generated from inflorescence (NaNakorn *et al*., 1998; Keshavachandran and Khader, 1997).

Improvement in vetiver plant, such as herbicide resistance and quality, have been generally achieved through cell selection (Prasertsongskun *et al*., 2002). New possibilities for improving vetiver may be provided by protoplast technology. Protoplast is a plant cell from which the cell wall has been removed. Therefore, DNA can be taken up directly by transformation involving somatic hybridization. The success of protoplast isolation depends on the condition of the tissue and the combination of enzyme being used (Galbraith *et al*.; 1984; Rasheed *et al*., 1990). The effect of cell densities for protoplast development was also reported (Kuchuk *et al*., 1998; Saito and Suzuki, 1999). Protoplasts from cell suspension and leaves are the most commonly obtained and usually regenerate into dividing cells. There are reports on isolation and culture of protoplasts from leaves and suspension in monocots. Karim and Adachi (1997) reported the successful isolation and culture of protoplast of *Allium cepa* from cell suspension culture. Kisaka *et al*. (1998) isolated protoplast of barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) and successfully obtained fusion product by electrofusion.

To date, there is no report on vetiver regeneration from protoplasts. In this paper, the author reports an attempt to isolate and culture protoplasts of vetiver Surat Thani germplasm.

Materials and Methods

Plant materials and initiation of cell suspension culture

Vetiveria zizanioides Nash Surat Thani germplasm was used in this experiment. Inflorescence was surface-sterilized by soaking in 70%

ethyl alcohol for 5 min then cut into small pieces and transferred to solid Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 15 µM 2,4-D. After three weeks of culture calli were transferred to solid MS medium supplemented with 10 µM 2,4-D. In this medium, compact yellow and friable embryogenic calli were formed. Only compact calli were selected and subsequently cultured in liquidified basal N_6 medium supplemented with 10 µM 2,4-D, 10 mM proline and 20 g/l sucrose (pH 5.8) (Prasertsongskun, 2003). The cultures were grown in 125-ml Erlenmeyer flasks on a gyratory shaker with an agitation of 110 rpm, under a 16-h photoperiod, a light intensity of 50 $\mu Em^{-2}s^{-1}$ and a temperature of 25±2°C. Cell suspension cultures of vetiver were established from the callus within 6 months. The cell suspension cultures were transferred to the same liquid N_6 medium and subcultured weekly.

Protoplast isolation

Protoplasts were isolated from 6-month-old suspension culture of vetiver which had been subcultured at weekly intervals. Four days after subculture, the cells from suspension cultures were used for isolation of protoplast. In this experiment, the enzyme solution was filter-sterilized and the washing solution was sterilized by autoclaving at 1.07 kg/cm^2, 121°C for 15 minutes. One milliliter packed cell volume (PCV) of cell suspensions were incubated in 4 ml of filter-sterilized enzyme solution consisting of various enzymes: cellulase Onozuka R10 (1.0, 2.0 and 3.0%), macerozyme R10 (1.0, 2.0 and 3.0%) and pectinase (0.5, 1.0 and 1.5%) (Table 1). The enzymes were dissolved in 0.4 M mannitol and 7 mM $CaCl_2.2H_2O$ (pH 5.8). The incubation was performed on a gyratory shaker set at 50 rpm for 10 h in the dark at 25± 2 °C. Protoplasts were filtered through 100-µm nylon mesh and washed twice with washing solution [0.4 M mannitol and 7 mM $CaCl_2.2H_2O$] by centrifugation at 500 rpm for 5 min. The protoplasts were purified by floating on a 21% (w/v) solution of sucrose and centrifuged at 500 rpm for 5 min, and then washed once with the washing solution. Protoplasts were counted using a haemacytometer slide and the density was adjusted to culture in various types of culture media.

Protoplast culture

N_6 medium containing 2% (w/v) sucrose was used as basal medium. The effects of different combinations of 2,4-D (0.5, 1.0 mg/l) and BA (0, 0.5, 1.0, 2.0 mg/l) on cell division were examined. Protoplasts at a density of 1×10^5 protoplasts/ml were cultured in these media and placed in 6×1.5 cm steriled Petri dishes. The effects of type of culture medium and protoplast density on division of protoplast were observed. Protoplasts were cultured in liquid and solid N_6 medium and comprising 1×10^5 and 2×10^5 protoplasts/ml. For liquid medium, protoplasts were suspended in 3 ml of the medium and cultured as thin layer. The solid medium was solidified by 0.15% Gelrite (Sigma). The Petri dishes were sealed with parafilm during the culture period. All cultures were maintained at 25±2°C in the dark. Dividing protoplasts were counted.

Experimental design and statistical analysis

This experiment was conducted in a completely randomized design (CRD) with five replications. Protoplast yields were counted by haemacytometer slide and the average number of protoplasts per ml PCV calculated. Dividing protoplasts were calculated by finding the number of cells showing division. Duncan's multiple range test (DMRT) was used for the statistical evaluation of experimental data. Standard deviation (SD) and levels of significance (*P*) are presented in the tables.

Results

Protoplast isolation

Maximum protoplast yields from suspension cultures were 8.4×10^4 protoplasts/ml PCV. It was clearly seen that all combinations of cellulase Onozuka R10 and macerozyme R10 in the absence of pectinase gave low protoplast yield. Addition of pectinase at 0.5% promoted a higher

Table 1. Effect of various enzyme combinations on yield of protoplasts of vetiver.

Enzyme (%)			Yield of protoplasts (protoplasts/ml PCV)±SD
Cellulase Onozuka R10	Macerozyme R10	Pectinase	
1.0	1.0	-	-
2.0	1.0	-	$0.2\times10^4\pm0.0$c
2.0	2.0	-	$0.4\times10^4\pm0.4$c
2.0	2.0	0.5	$8.4\times10^4\pm0.5$a
3.0	2.0	1.0	$3.2\times10^4\pm2.4$b
2.0	3.0	1.5	$1.0\times10^4\pm1.3$bc

Means followed by the same letter are not statistically significant at $P<0.05$ according to Duncan's multiple range test (DMRT). Each value is the mean of five replicates.
SD = Standard deviation

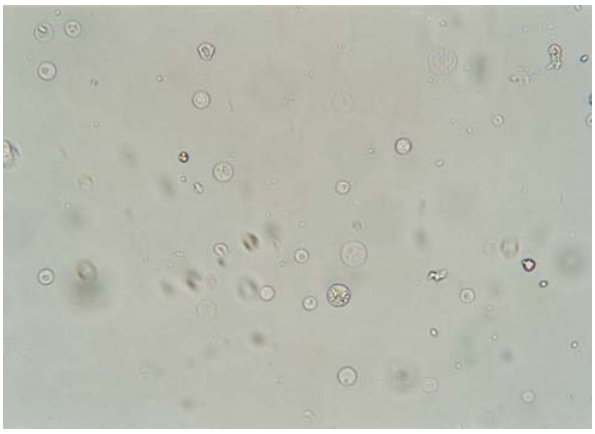

Figure 1. Isolated protoplasts from vetiver cell suspension (100×).

yield of protoplasts. Concentration of pectinase higher than 0.5% did not provide high yield of protoplasts (Table 1). There was a great variation in protoplast size. The size of the protoplasts ranged from 10 µm to 25 µm (Figure 1).

Protoplast culture

The effect of various combinations of plant growth regulators in the initial protoplast culture was tested, and the results are shown in Table 2. The suitable medium for the culture of vetiver protoplasts was liquid N_6 medium supplemented with 1.0 mg/l 2,4-D and 0.5 mg/l BA. Percentage of protoplast division in this medium was 5.0%.

Table 2. Effects of combination of growth regulators on division of protoplasts of vetiver in liquid N_6 medium.

Plant growth regulators (mg/l)		Protoplast division (%)±SD
2,4-D	BA	
0.5	0	0.0±0.0a
	0.5	1.1±2.5a
	1.0	2.2±3.1a
	2.0	1.1±2.5a
1.0	0	1.3±3.0a
	0.5	5.0±2.9a
	1.0	2.5±3.5a
	2.0	2.2±3.1a

Means followed by the same letter are not statistically significant at $P<0.05$ according to Duncan's multiple range test (DMRT). Each value is the mean of five replicates.
SD = Standard deviation

Protoplast culture was monitored using two different methods, namely liquid and solid media. Mitotic division was observed only in liquid culture. With protoplast densities of 1×10^5 and 2×10^5 protoplasts/ml, the percentage of cell divisions were 5.1 and 1.3%, respectively (Table 3). The density of 1×10^5 protoplasts/ml gave optimum protoplast division (5.1%), but this response was not statistically different from the response obtained (1.3%) with density of 2×10^5 protoplasts/

Table 3. Effect of culture method on division of protoplasts of vetiver.

Culture method	Protoplast density (protoplasts/ml)	Protoplast division (%)±SD
Liquid medium	1×10^5	5.1±2.9a
	2×10^5	1.3±3.0a
Solid medium	1×10^5	0.0±0.0b
	2×10^5	0.0±0.0b

Means followed by the same letter are not statistically significant at $P<0.05$ according to Duncan's multiple range test (DMRT). Each value is the mean of five replicates.
SD = Standard deviation

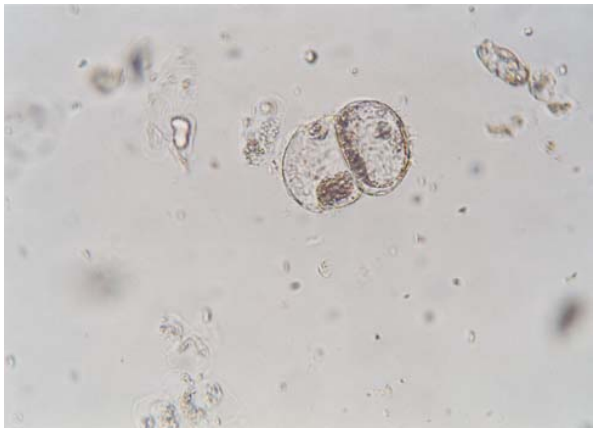

Figure 2. First division of vetiver protoplast at 3 days after culture (200×).

ml. The first cell division occurred 3 days after culture (Figure 2). Microcolony was not obtained. Protoplasts plated in solid medium failed to divide.

Discussion

The efficiency of protoplast isolation and culture depends on many factors, such as the enzyme mixture, the presence of growth regulator and the length of time after subculture (Assani *et al*., 2001). Protoplast release with the enzyme mixture (2% cellulase Onozuka R10, 2% macerozyme R10 and 0.5% pectinase) occurred within 10 h. Pectinase was required to increase protoplast yield. The optimum combination of plant growth regulators for cell division initiation was 1.0 mg/l 2,4-D and 0.5 mg/l BA on N_6 medium. This result indicated that combination of BA and 2,4-D in high concentration inhibited protoplast division. This result was consistent with earlier findings that the combined optimal auxin and cytokinin were relatively effective for cell division in petal protoplast of *Petunia hybrida* (Oh and Kim, 1994), and in cell suspension protoplast of *Allium cepa* (Karim and Adachi, 1997). Another important factor for protoplast culture is the culture system. In these experiments protoplasts were cultured either in liquid and solid N_6 medium comprising 1×10^5 and 2×10^5 protoplasts/ml. Division of protoplasts obtained in liquid N_6 medium at optimal density was 1×10^5 protoplasts/ml. The density of protoplasts influenced the initiation of cell divisions, as has been reported in oat by Hahne *et al*. (1990). The suspension-derived protoplasts of vetiver did not divide in Gelrite. In contrast to published data (Kisaka *et al*., 1998) the same gelrite was successfully used for protoplast culture. There were some reports that agarose and phytagel have been used to improve protoplast culture in *Medicago* sp. and *Garcinia atroviridis* Griff., respectively (Gilmour *et al*., 1987; Te-chato, 1997).

To the author's knowledge the isolation and culture protoplasts of vetiver have been reported here for the first time. The regeneration of protoplast of vetiver should be further investigated.

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References

Assani, A., Haicour, R., Wenzel, G., Côte , F., Bakry, F., Foroughi-Wehr, B., Ducreux, G., Aguillar, M.-E. and Grapin, A. 2001. Plant regeneration from protoplasts of dessert banana cv. Grande Naine (*Musa* spp., Cavendish sub-group AAA)

via somatic embryogenesis. Plant Cell Rep. 20: 482-488.

Galbraith, D.W., Afonso, C.L. and Harkins, K.R. 1984. Flow sorting and culture of protoplasts: Conditions for high-frequency recovery, growth and morphogenesis from sorted protoplasts of suspension cultures of nicotiana. Plant Cell Rep. 3: 151-155.

Gilmour, D.M., Davey, M.R. and Cocking, E.C. 1987. Plant regeneration from cotyledon protoplasts of wild *Medicago* species. Plant Sci. 48: 107-112.

Hahne, B., Lörz , H. and Hahne, G. 1990. Oat mesophyll protoplasts: their response to various feeder cultures. Plant Cell Rep. 8: 590-593.

Karim, M.A. and Adachi, T. 1997. Cell suspension, isolation and culture of protoplasts of *Allium cepa*. Plant Cell Tiss. and Org. Cult. 51: 43-47.

Keshavachandran, R. and Khader, M.A. 1997. Growth and regeneration of vetiver (*Vetiveria zizani oides* (L.) Nash) callus tissue under varied nutritional status. **In** Biotechnology of Spices, Medicinal and Aromatic Plants (eds. Edison, S., Ramana, K.V., Sasikumar, B. and Babu, K.N.) pp. 60-64,. Proceedings of National Seminar on Biotechnology of Spices and Aromatic Plants. Calicut, India.

Kisaka, H., Kisaka, M., Kanno, A. and Kameya, T. 1998. Intergeneric somatic hybridization of rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.) by protoplast fusion. Plant Cell Rep. 17: 362-367.

Kuchuk, N., Herrmann, R.G. and Koop, H.-U. 1998. Plant regeneration from leaf protoplasts of evening primrose (*Oenothera hookeri*). Plant Cell Rep. 17: 601-604.

Mucciarelli, M., Gallino, M., Scannerini, S. and Maffei, M. 1993. Callus induction and plant regeneration in *Vetiveria zizanioides*. Plant Cell Tiss. and Org. Cult. 35: 267-271.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15: 473-497.

NaNakorn, M. Surawattananon, S., Wongwattana, C., Namwongprom, K. and Suwannachitr, S. 1998. *In vitro* induction of salt tolerance in vetiver grass (*Vetiveria zizanioides* Nash). J. Weed Sci. Tech. 43: 134-137.

Oh, M.-H. and Kim, S.-G. 1994. Plant regeneration from petal protoplast culture of *Petunia hybrida*. Plant Cell Tiss. and Org. Cult. 36: 275-283.

Prasertsongskun, S. 2003. Plant regeneration from callus culture of vetiver (*Vetiveria zizanioides* Nash). Songklanakarin J. Sci. Technol. 25: 637-642.

Prasertsongskun, S., Sangduen, N., Suwanwong, S., Santisopasri, V. and Matsumoto, H. 2002. Increased activity and reduced sensitivity of glutamine synthetase in glufosinate-resistant vetiver (*Vetiveria zizanioides* Nash) cells. Weed Bio. Manage. 2: 171-176.

Rasheed, J.H. Al-Mallah, M.K., Cocking, E.C. and Davey, M.R. 1990. Root hair protoplasts of *Lotus corniculatus* L. (birdsfoot trefoil) express their totipotency. Plant Cell Rep. 8: 565-569.

Saito, A and Suzuki, M. 1999. Plant regeneration from meristem-derived callus protoplasts of apple (*Mulus* × *domestica* cv.'Fuji'). Plant Cell Rep. 18: 549-553.

Sayamanonta, R., Nanakon, W., Chalothorn, C., Thiraporn, R. and Nanagara, T. 1996. Integrated vetiver programme-the Thai experience, pp. 6-8. **In** Report of the first International Conference on Vetiver: A Miracle Grass. Feb. 4-8 th., 1996. Chiang Rai Thailand.

Te-chato, S. 1997. Isolation and culture of protoplast of somkhag (*Garcinia atroviridis* Griff.) to microcolony. Songklanakarin J. Sci. Technol. 19: 255-262.