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Explant-nurse Culture of Protoplasts and Plant Regeneration in the Radish

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Explant-nurse culture experiments were carried out to raise the efficiency of protoplast culture of radish (*Raphanus sativus* L. cv. Shirohime) using the cotyledon, root and callus as nurse sources. Protoplasts were isolated by incubating cotyledon and leaflet slices in an enzyme solution for 3 h at a shaking rate of 40 rpm. The cotyledon-nurse was most efficient for cell division and colony formation in Murashige and Skoog (MS)[5] medium, followed by the root-nurse and then the callus-nurse. The rate of cell division was similar to a measure of cell division, the plating efficiency of cruciferae[1]. However, cell division and colony formation was very low without nurse-explants. Shoots were formed from yellow and/or green calli during the 30-day period after transfer onto regeneration media supplemented with 0.1 mg/*l* of benzyladenine (BA) plus 2.0 mg/*l* kinetin and 2.0 mg/*l* zeatin plus 2.0 mg/*l* kinetin. Their rate of shoot regeneration was 18.5% and 13.2%, respectively. However, the rate of shoot differentiation decreased in the media containing BA and zeatin in combination with other concentrations of kinetin. These results indicate that the effects of BA and zeatin on shoot differentiation are closely related to the concentration of kinetin. The present results offer basic information that may be useful for improving protoplasts culture and plant regeneration in the radish.

Introduction

Establishment of a plant regeneration procedure from protoplasts is required for efficiently manipulating somatic hybridization or genetic transformation. The radish is one of the most important vegetables in the world. However, there is limited information on protoplast culture[1,2], and plant regeneration from protoplast-derived calli[1] in the radish. Variations in colony and shoot formation from protoplast-derived calli have been reported for different radish cultivars[1]. Shoot formation occurred in only one cultivar (Moriguchi) out of eight examined[1]. These results contrast with those of *Brassica oleracea* species[1]. Thus, an alternative strategy is required to improve the efficiency of protoplast culture and the rate of plant regeneration in the radish.

The agarose bead nurse culture method has been adopted for the protoplast culture of other plants[3,6,9]. However, information on the effects of agarose nurse culture in combination with other nurse sources on radish protoplast culture is lacking. This, in turn, may require the improvement of nutrient constitutions and hormone combinations in the culture medium.

The present experiments were conducted to examine the effects of nurse sources, including the cotyledon, root and callus, on protoplast culture, and the effects of hormone combinations in the culture medium on plant regeneration in the radish.

Materials and Methods

1. *Plant and nurse materials*

Seeds of the radish cultivar, Shirohime were sterilized in 1% sodium hypochlorite for 20 min, and sown on MS medium containing 1 mg/*l* α-naphthaleneaceticacid (NAA), 0.5 mg/*l* BA, 2% sucrose and 0.8% agar at pH 5.8. Cotyledons and leaflets were taken from 3- to 10-day-old seedlings that were grown under a fluorescence light intensity of 2000 lux and 16 h day-length at 20°C.

Seedlings were also grown under sterile conditions, and the above-mentioned light and temperature conditions. Nurse cotyledons, that were sampled from 5- to 8-day-old seedlings, were *ca.* 1.3 cm in width and *ca.* 0.7 cm in length. Nurse roots of *ca.* 2 cm in length were also sampled from the same seedlings. Nurse calli were induced from the sterilized seeds on MS medium supplemented with 2 mg/*l* 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg/*l* BA. The calli were subcultured on the same MS medium three times at intervals of 3 weeks, and the nurse-calli were yellow and relatively hard.

2. *Protoplast isolation*

The cotyledon and leaflet (*ca.* 1 g fresh weight) from young seedlings were cut into 2 mm pieces wide, and incubated in 20 m*l* of liquid 1/2 MS solution containing 0.5% cellulase Onozuka RS (Yakult, Tokyo, Japan), 0.05% macerozyme R-10 (Yakult, Tokyo, Japan), 0.01% pectolyase Y-23 (Kikkoman, Tokyo, Japan), 0.5 M mannitol and 1% sucrose. The solution was shaken at the rate of 40 rpm at 25°C for 3 h in the dark. The released protoplasts were filtered through a nylon sieve of 25 μm pore size, and then washed 3 times with 0.4 M mannitol solution, followed by centrifugation at a rate of 600 rpm for 4 min.

3. *Protoplast culture with nurses*

The liquid MS medium for protoplast culture consisted of 1.0 mg/*l* 2, 4-D, 1.0 mg/*l* NAA, 0.5 mg/*l* BA, 0.4 M mannitol, 1.0% sucrose and 500 mg/*l* casamino acid. The solid MS medium contained 1% (w/v) agarose (Seaprep agarose, FMC, Bioproducts, Rockland, USA) in the above-mentioned liquid MS medium. Explants of cotyledon, root, and callus were used as the nurse for protoplast culture.

Protoplasts were suspended and cultured in 5 m*l* of liquid MS medium at a density of 5×10^6/m*l* in a plastic Petri dish of 6 cm in diameter. Agarose-solidified protoplasts were also cultured at the same density in 5 m*l* of liquid MS medium in the same size Petri dish.

The agarose-solidified MS medium was cut into eight blocks. To initiate cell division, two blocks were placed in each of six Petri dishes, three of which contained 5 m*l* of liquid MS, and three of which contained 5 m*l* of B medium. Thereafter, the MS medium was replaced with 2/3 MS medium, and the B medium was replaced with C medium for colony formation after 7 days of incubation for both media. The B and C media[7] are the modified MS media. The B medium was supplemented with 1 mg/*l* NAA and 0.25 mg/*l* 2, 4-D, and the C medium was supplemented with 0.2 mg/*l* NAA, 1 mg/*l* BA, 0.1 mg/*l* 2, 4-D and 0.02 mg/*l* gibberellic acid (GA_3). These media have been recommended for the protoplast culture of the radishes[1,7].

After 5 days incubation, 2- to 4-cell clusters were observed, and these developed into colonies or mini-calli of 0.1 to 0.2 mm in diameter by 15 days. The rate (%) of cell division and colony formation was transformed into Arcsin$\sqrt{\ }$% for calculating the mean and standard error.

4. *Shoot and plant regeneration*

Protoplast-derived colonies of *ca.* 2 mm in diameter, *i. e.*, mini-calli that had been subcultured 5 times over a 4-month period without selection, were transferred onto modified MS medium. The

modified MS medium contained 0.5% agarose and 3% sucrose with varying combinations and concentrations of the hormones, zeatin, kinetin and BA (described in **Table 3**). Callus-derived shoots, that attained more than 2 cm in length, were transferred onto a hormone-free MS medium, where they efficiently formed roots. The plantlets were then acclimatized.

Results

1. *Cell division and colony formation*

The effects of the culture media and nurse sources on cell division are shown in **Table 1** and **Fig. 1**. The cotyledon-nurse was most efficient for cell division, followed by the root-nurse in liquid MS medium, and then the non-nurse (**Fig. 1-A, B** and **C**). However, adhesion of protoplasts around the root-nurse occurred in liquid medium (**Fig. 1-B**). Similar results concerning the nurse effect on cell division were obtained in the agarose-medium, although the rate of cell division was lower than in the liquid medium (**Table 1**).

The effects of changes of both the media (MS to 2/3 MS and B to C) were examined with the nurse-effects on the rates of protoplast division and colony formation. However, the nurse-effects on the rates of protoplast division and colony formation were difficult to be examined in the liquid medium due to the coagulation of protoplasts. Thus, the nurse-effects on protoplast division and colony formation were examined using the agarose-solidified protoplasts.

The rate of cell division was significantly higher in the MS medium than in B medium regardless

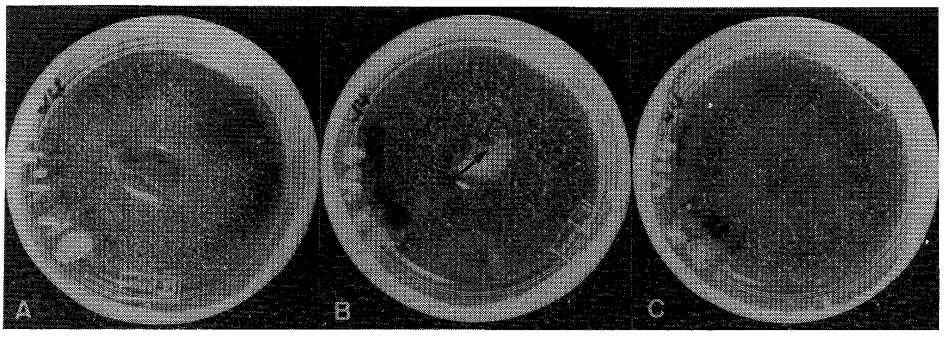

Fig. 1 Effect of nurse sources on protoplast culture of the radish.
A: Cotyledon-nurse.
B: Root-nurse (the arrow indicates adhesion of protoplasts to root-nurse).
C: Non-nurse.
The liquid MS medium was replaced 3 times at intervals of *ca.* 10 days, and all protoplasts were taken after 30 days incubation in order to present a clear image.

Table 1. Effects of culture medium and nurse source on cell division.

Nurse	Liquid MS medium			Agarose-solidified MS medium		
	No. of cells observed*1	No. of dividing cells*2	Rate of dividing cells	No. of cells observed*1	No. of dividing cells*2	Rate of dividing cells
	Mean SE	Mean SE	Mean SE (Arcsin$\sqrt{\ }$%)	Mean SE	Mean SE	Mean SE (Arcsin$\sqrt{\ }$%)
Cotyledon	49.8±1.8	23.8±1.1	49.9±1.9	49.2±2.7	15.3±0.7	32.2±2.4
Root	53.8±2.5	17.7±1.3	33.6±2.4	48.3±1.0	10.0±1.2	20.8±2.3
Non-nurse	50.3±2.7	1.3±0.5	2.6±1.0	49.3±2.4	3.3±1.1	6.7±2.1

*1 and *2 Data were recorded 5 days after incubation. Standard errors were calculated on the basis of 6 measurements from 2 agarose blocks in 3 flasks. Data are based on the number of viable cells counted in a 10 (ocular) ×20 (objective) microscopic field of each agarose block.

Table 2. Effects of nurse source and culture medium constitution on cell division and colony formation.

Nurse	Cell division*1		Colony formation*2	
	Culture medium			
	MS	B	2/3 MS	C
	Arcsin$\sqrt{\ }$%			
	Mean SE	Mean SE	Mean SE	Mean SE
Cotyledon	43.0±3.2	23.5±3.1	37.0±1.9	18.5±1.2
Root	37.7±4.3	17.0±2.5	30.3±2.1	15.0±2.4
Callus	26.5±2.5	7.4±1.4	14.3±2.1	4.4±1.1
Non-nurse	11.0±1.8	1.2±0.6	1.3±0.6	0.0

*1 After 5 days incubation.

*2 After 15 days incubation.

Standard errors were calculated on the basis of 6 measurements from 2 agarose blocks in 3 flasks. Data are based on the number of viable cells counted in a 10(ocular)×20(objective) microscopic field of each agarose block.

of nurse sources. The rate of colony formation was also significantly higher in the 2/3 MS medium than in C medium regardless of nurse sources (**Table 2**). The rate of cell division and colony formation was significantly higher in the cotyledon- and root-nurse cultures than in the callus- and non-nurse cultures. In addition, the rate of cell division and colony formation tended to be higher in the cotyledon-nurse than in root-nurse, and furthermore was significantly higher in the callus-nurse than in the non-nurse (**Table 2**).

2. *Callus formation and plant regeneration*

Protoplast-derived colonies of 0.1 to 0.2 mm in diameter (**Fig. 2-A**) were incubated in the agarose-

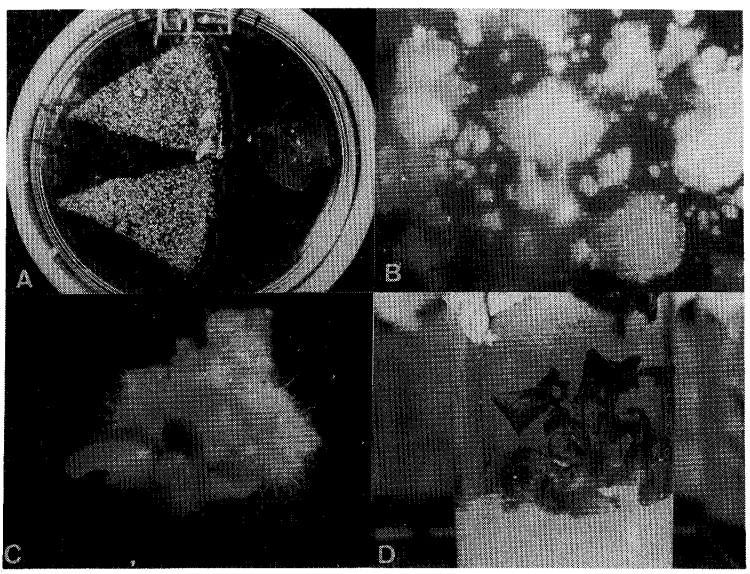

Fig. 2 Plant regeneration from protoplast-derived calli via shoot formation in the radish.
A: Protoplast-derived colonies with cotyledon-nurse 3 weeks after protoplast incubation.
B: Mini-calli 5 weeks after protoplast incubation.
C: Shoots 5 weeks after transfer into regeneration medium.
D: Plantlets 3 months after transfer into regeneration medium.

Table 3. Effects of hormone combinations on shoot regeneration from protoplast-derived calli*[1].

Hormone combination (mg/*l*)	No. of calli inoculated	No. of calli with shoots	%
Zeatin 0.5	131	0	
Zeatin 1.0	130	0	
Zeatin 1.0+Kinetin 1.0	134	0	
Zeatin 1.0+Kinetin 2.0	145	9	6.2
Zeatin 1.0+Kinetin 3.0	129	0	
Zeatin 1.0+Kinetin 4.0	130	0	
Zeatin 2.0	136	0	
Zeatin 2.0+Kinetin 1.0	146	4	2.7
Zeatin 2.0+Kinetin 2.0	129	17	13.2
Zeatin 2.0+Kinetin 3.0	134	10	7.5
Zeatin 2.0+Kinetin 4.0	140	0	
BA 0.5	127	0	
BA 1.0	134	0	
BA 1.0+Kinetin 1.0	126	4	3.4
BA 1.0+Kinetin 2.0	130	24	18.5
BA 1.0+Kinetin 3.0	138	13	9.5
BA 1.0+Kinetin 4.0	132	0	
BA 2.0	133	0	
BA 2.0+Kinetin 1.0	130	0	
BA 2.0+Kinetin 2.0	130	0	
BA 2.0+Kinetin 3.0	141	0	
BA 2.0+Kinetin 4.0	136	0	

*[1] Calli were inoculated in MS medium containing 0.5% agarose, 3% sucrose and 0.1 mg/*l* NAA. Data were recorded after 30 days incubation.

solidified MS medium containing 2.0 mg/*l* 2, 4-D for callus proliferation. The colonies developed to yellow and/or green mini-calli of 6 to 8 mm in diameter after 2 weeks (**Fig. 2-B**). After 30 days incubation, shoots differentiated in the media supplemented with either 2.0 mg/*l* zeatin or 1.0 mg/*l* BA in combination with 1.0, 2.0 and 3.0 mg/*l* kinetin (**Table 3**). The regenerated shoots formed roots on hormone-free MS medium (**Fig. 2-C**), and the plantlets (**Fig. 2-D**) were acclimatized according to ordinary procedures.

Discussion

The cotyledon-nurse was most efficient for radish protoplast culture, followed by the root-nurse. The cotyledon- and root-nurse can also be applied in the liquid cultures of protoplasts from *Brassica rape* and *B. oleracea* (in the preliminary experiments, data not shown). The rate of cell division was comparable to the plating efficiency in *Raphanus*[1]. Cultivar variations in the rates of colony and shoot formation were reported[1,2] using B and C media that were recommended for protoplast culture of cruciferae[1]. However, the rate of cell division in cotyledon- and root-nurse culture using MS medium was similar to the plating efficiency of cruciferae, *Raphanus* species and other crops[1]. In addition, the rate of colony formation appeared to correspond to that of cell division. This fact may indicate that the nurse-effect on the rate of colony formation is expressed through the nurse-effect on the rate of cell division.

Addition of BA, GA_3 and zeatin into the culture medium stimulated shoot formation[4,8]. However, the effects of BA and zeatin on shoot formation appeared to be enhanced by the addition of kinetin, although its effect on shoot formation was closely related to the concentration of kinetin

in radish. Thus, a relatively high effectiveness was obtained only with the combinations of 2.0 mg/*l* zeatin plus 2.0 mg/*l* kinetin or 1.0 mg/*l* BA plus 2.0 mg/*l* kinetin.

Generally, kinetin or endogenous cytokinins are the elite agents for inducing organogenesis from calli. The present results also confirmed the promising role of kinetin. However, the rate of shoot formation is still low, *i. e.*, in the range of 13 to 18% in the present results, although it is in the same range reported for the radish cultivar, Moriguchi[1]. Thus, it may be necessary to examine the combination of kinetin with other hormones to raise the rate of shoot formation.

The present results provide basic information for use in current studies aimed at improving the rate of colony and shoot formation, and as well as improving plant regeneration. The next goal is to improve somatic hybridization and genetic transformation based on the present results.

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《和文要約》

外植体を用いたダイコンのプロトプラストのナース培養と植物体再生

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子葉，根及びカルスをナースとして用い，ダイコン(品種：白姫)のプロトプラスト培養を行った．プロトプラストは，無菌的に発芽させた幼植物体の子葉及び幼葉から酵素処理によって単離した．酵素処理は，40 rpm で3時間振盪を行った．その結果，MS 培地を用いて子葉，根をナースとした場合の細胞分裂率はこれまで報告された十字花科作物などの結果とほぼ同じであった．また，子葉及び根をナースとした場合の細胞分裂率及びコロニー形成率はカルス及び無ナースの場合よりも有意に高い値を示した．さらに，子葉ナースは根ナースよりも細胞分裂率及びコロニー形成率が高い傾向を示し，カルスナースは無ナースよりも細胞分裂率及びコロニー形成率が高い傾向を示した．

コロニー由来の黄色ないし緑色カルスから，培養30日後に zeatin 2.0 mg/*l*＋kinetin 2.0 mg/*l* のホルモンの組み合わせで13.2%，BA 1.0 mg/*l*＋kinetin 2.0 mg/*l* のホルモンの組み合わせで18.5% の幼芽が分化した．同じ zeatin 及び BA 濃度と kinetin 濃度1.0及び3.0 mg/*l* の組み合わせでは，幼芽分化率は10% 以下に低下した．このことは，幼芽形成に有効であるとされている zeatin と BA の効果が kinetin の濃度によって影響されることを示唆している．

本研究の結果は，作物の中で比較的に困難とされているダイコンのプロトプラスト培養，さらに植物体再生の改良に知見を与えるものである．今後の課題は，これらの知見を基にして細胞融合，形質転換植物体の育成を検討することである．