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Citation	Journal of the Faculty of Agriculture, Hokkaido University, 65(1), 119-125
Issue Date	1991-03
Doc URL	http://hdl.handle.net/2115/13112
Type	bulletin (article)
File Information	65(1)_p119-125.pdf

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J. Fac. Agr. Hokkaido Univ., Vol. 65, Pt. 1 : 119~125 (1991)

PLANT REGENERATION FROM MESOPHYLL PROTOPLASTS OF BRUSSELS SPROUTS (*Brassica oleracea* var. *gemmifera* Z.)

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Received December 26, 1990

Introduction

Brassica is one of the most important vegetables cultivated at present in Japan and has a number of cultivated species. Recently, protoplast has come into general use in plant breeding. Some examples were reported as to establishment of somatic hybrids among *Brassica* plants[11]. Plant regeneration from protoplast was reported in cabbage[1,3,6,8], broccoli[4,10], cauliflower[4] and other species[9,13].

Brussels sprouts is one of the subspecies of *B. oleracea* and characterized by having many small bulbs produced at each node of stalk. There is a possibility to breed a new vegetable through cell fusion between Brussels sprouts and another *Brassica* plant. However there are no reports on the culture of protoplasts in Brussels sprouts.

It is important to select growing materials at appropriate physiological stage of donor plant for protoplast division. Sterilized plants of Brussels sprouts can be cultured under uniform condition.

The present work was conducted to determine the most appropriate material, condition for protoplast culture and plant regeneration in Brussels sprouts.

Materials and Methods

1. Plant materials

The cultivar 'Wase-komochi-kanran' was used in the present work. Seeds were surface-sterilized with 70% ethanol for 1 minutes and 1% sodium hypochlorite solution for 15 minutes, then washed four times with sterile distilled water. The sterilized seeds were germinated on MS solid medium supplemented with 10 g/l sucrose and 8 g/l agar, and grown at 25°C under 16 h photoperiod with

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3,000 lux illumination by fluorescent lamps. Cotyledons, hypocotyls of 14 days and young leaves of 1 month seedlings were used for protoplast isolation.

2. Enzyme solution

The enzyme solution for maceration contained 0.1% Pectolyase (Seishin Seiyaku Co., LTD.), 0.5% Cellulase Onozuka RS (Kinki Yakult Co., LTD.), CPW salts solution and 0.5 M mannitol. The pH of this solution was adjusted to 5.8 with 0.1 N NaOH or HCl. The enzyme solution was sterilized by filtration through a 0.22 μm membrane filter.

3. Protoplast isolation

Young leaves, cotyledons and hypocotyls were sliced into thin pieces with a razor blade. These pieces, 1 gram in flesh weight for each plant part, were soaked 10 ml enzyme solution in a screw-capped 50 ml flask for 2 to 3 hours at 27° C. The crude protoplast suspension was filtrated through a 45 μm nylon mesh. The filtrate was centrifuged at 100 x g for 3 minutes by adding 0.5 M sucrose. The precipitate was extracted by a pastuer pipette and washed three times in Murashige & Skoog (MS) liquid medium with 0.5 M mannitol.

4. Protoplast culture

Basal medium was composed of half strength MS inorganic salts without NH_4NO_3 and KNO_3, half strength MS vitamin, 10 g/l sucrose and 0.5 M mannitol. A quarter of regular NH_4NO_3 and KNO_3 concentration in MS medium were added as a nitrogen source,. To the basal medium, four levels of α-naphthaleneacetic acid (NAA), one level of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and three levels of 6-benzyladenine (BA) were added as shown in Table 1. The pH was ajusted to 5.8. All protoplasts were cultured in a thin layer of liquid or agarose medium at a density of 3 to 5×10^4 cells/ml in microplate (1 cm in diameter, Corning Co., LTD.). The microplates were sealed with parafilm and placed at 25°C under dark condition.

5. Plant regeneration

Some calli induced from mesophyll protoplasts were transferred to MS meium supplemented with 0.5 mg/l 2, 4-D and 3.0 mg/l kinetin after 35 days. The others were transferred to the medium supplemented 3 combinations of plant growth regulators, 0.035 mg/l Gibberellin (GA_3) and 1.0 mg/l BA, 0.1 mg/l indole -3-acetic acid (IAA) and 1.0 mg/l BA, and 0.5 mg/l zeatin after 71 days of incubation. Then, the calli differentiating shoots were transferred to MS basal medium without plant growth regulator for rooting.

6. Transferring to soil

After having been washed in running water in order to remove the components of the medium such as sugar and agar from the roots, regenerated plants were transferred to small pots. These plants were grown in an artificial climate chamber at 20°C with a 16 hour day length at 8,000 lux for two weeks. The plants with approximate 10 leaves were transferred to a greenhouse and grown there.

Table 1. Effects of NAA, 2,4-D and BA on mesophyll protoplast division, colony and callus induction.

NAA (mg/l)	2,4-D (mg/l)	BA (mg/l)	Protoplast division[z]	Colony formation[y]	No. of calli formed[x]
0.1	0	0	−	−	−
0.1	0	0.1	−	−	−
0.1	0	1.0	−	−	−
0.5	0	0	−	−	−
0.5	0	0.1	+	+	4
0.5	0	1.0	+	+	2
1.0	0	0	−	−	−
1.0	0	0.1	++	+	4
1.0	0	1.0	+	+	3
5.0	0	0	−	−	−
5.0	0	0.1	−	−	−
5.0	0	1.0	−	−	−
0	0.1	0.1	++	+	4

z;(−) indicates no protoplast division and 0 to 20 (+) or more than 20 (++) protoplasts divided per microplate after 7 days of incubation.
y;After 15 days of incubation
x;Average number of induced calli in a microplate was described after 35 days of incudation.

Morphological variation was investigated in regenerated plants after 2 months.

Results

1. Protoplast isolation

Protoplast isolation was influenced by the part and the age of plant materials. Viable protoplasts were obtained from cotyledons aged 14 days and fully expanded young leaves of one month old. Hypocotyls did not give active protoplasts.

2. Protoplast division

Mesophyll protoplasts isolated from young leaves were bred in the medium supplemented with NAA, 2, 4-D and BA, especially 2 combinations, 1.0 mg/l NAA and 0.1 mg/l BA and 1.0 mg/l 2, 4-D and 0.1 mg/l BA (Table 1). Cell division of protoplast occurred after 4 days of incubation (Fig. 1). Cell clusters consisting of about 20 cells were formed after 11 days. By gradually reducing the concentration of mannitol from 0.5 M to 0.1 M, some cell clusters developed into yellow compact calli of approximate 2 mm in size.

Protoplasts isolated from expanding young leaves, smaller than 1 cm in width, divided vigorously. On the other hand, protoplasts isolated from larger leaves did not divided. Besides, protoplast division was observed in the liquid

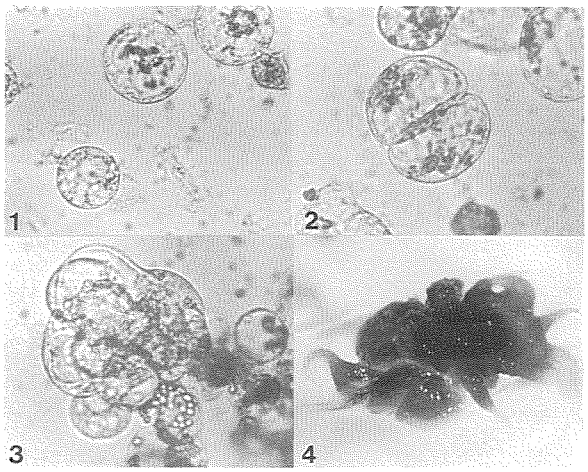

Fig. 1 Division and callus induction from mesophyll protoplast. 1, Isolated protoplast. 2, First cell division after 7 days of incubation. 3, Cell cluster formation after 9 days of incubation. 4, Callus formation after 2 months of incubation.

Table 2. Effects of NAA, 2,4-D and BA on division of protoplast isolated from cotyledon.

NAA (mg/l)	2,4-D (mg/l)	BA (mg/l)	Protoplast division[z]	Plating efficiency[y] (%)
0	0.1	0.01	+	−
0	0.1	0.1	+	−
0	1.0	0.1	++	10.7
0.1	0	0.01	+	−
0.1	0	0.1	−	−
1.0	0	0.1	++	3.7

z; (−) indicates no protoplast division and 0 to 20 (+) or more than 20 (++) protoplasts divided per microplate after 7days of incubation.
y; After 14 days of incubation.

medium and was not observed in the agarose medium.

In the protoplasts isolated from cotyledon, initial division was observed after 7 days of incubation (Table 2). Protoplasts divided continuously in the medium supplemented with 1.0 mg/l NAA and 0.1 mg/l BA, and in that with 1.0 mg/l 2, 4-D and 0.1 mg/l BA. The Plating efficiency was 3.7% in the former and 10.6% in the latter after 14 days of incubation. Callus formation was accelerated by reducing the concentration of mannitol to 0.25 M after 28 days of incubation.

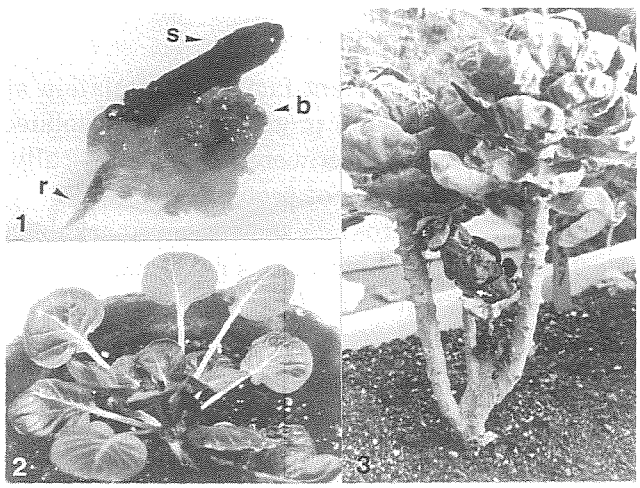

Fig. 2 Regeneration from the callus induced from mesophyll protoplast and regenerated plant.
1, Regeneration from the callus. Adventitious shoot (s), adventitious roots (r) and many adventitous buds (b) were differentiated. 2, Regenerated plant growing in a pot. 3, Regenerated plant with four stalks.

3. Regeneration

Rooting was observed in the calli induced from the mesophyll protoplasts of young leaves in the medium supplemented with 0.5 mg/l NAA and 0.1 mg/l BA after 35 days of incubation. But no plantlet was induced.

After 70 days of incubation, the calli induced in the medium supplemented with 1.0 mg/l NAA and 0.1 mg/l BA, or with 1.0 mg/l 2, 4-D and 0.1 mg/l BA were transferred to the medium for regeneration. Adventitious buds were formed only in MS medium supplemented with 0.035 mg/l GA_3 and 1.0 mg/l BA. Frequency of the adventitious bud formation was highly affected by the media for callus induction. Adventitious bud differentiation was observed in the calli developed in the medium with 1.0 mg/l 2, 4-D and 0.1 mg/l BA. These calli were cut into small pieces, and new shoots and roots developed from them in the medium without plant growth regulator (Fig. 2-1). The calli developed in the medium with 1.0 mg/l NAA and 0.1 mg/l BA differentiated the adventitious buds in the medium for regeneration. These buds, however, did not develop.

4. Variation in the regenerated plants

Three plants obtained from mesophyll protoplasts were successfully transferred to soil (Fig. 2-2). They produced elongated stalks and had some small bulbs at the node. One of them had four stalks (Fig. 2-3) and the others had two stalks.

Discussion

Material is one of the most important factors for isolation and division of protoplasts. A large number of viable protoplasts were isolated from young leaves and cotyledons in the present experiments. Physiologically young tissues are apparently effective in the isolation of protoplast.

Concentrations of auxin and cytokinin showed a remarkable effects on the division of protoplast isolated from both tissues mentioned above. It is considered that the medium supplemented with 1.0 mg/l NAA and 0.1 mg/l BA or with 1.0 mg/l 2, 4-D and 0.1 mg/l BA was appropriate for vigorous division of protoplasts.

The medium supplemented with 0.035 mg/l GA_3 and 1.0 mg/l BA was found to be an appropriate medium for regeneration. It is considerable that noteworthy GA_3 is a necessary plant growth regulator for adventitious bud differentiation in the callus induced from Brussels sprouts mesophyll protoplasts as other *Brassica* species[5,8].

The type of auxin in the initial medium had a strong effect on regeneration and 2,4-D is supposed to be an appropriate auxin for the culture of mesophyll protoplasts in Brussels sprouts. But, further works are necessary to determine the effect of auxin type on the culture of mesophyll protoplast.

The percentage of calli forming adventitious buds is still low. It was reported by Nishio *et al.* that the regeneration capacity of callus may be determined in an early stage of culture[8]. The present data supports this view.

Young leaves of donor plants were used as the material for protoplast culture in many plants[2], while some plants were successfully induced from cotyledon protoplasts[7], In our experiment, no plantlet was induced from cotyledon protoplasts. The cotyledon aged 14 days may be too senescent physiologically for this purpose. The appropriate age of the cotyledon for material must be investigated.

Some variations were reported in the regenerated plants from protoplasts in certain species[12]. Further work is required to establish the method of regeneration without variation in Brussels sprouts.

Summary

Numerous viable protoplasts of Brussels sprouts (*Brassica oleracea* var. *gemmifera* Z.) were isolated from cotyledons aged 14 days and expanding young leaves of 1 month old cultured *in vitro*. Vigorous protoplast division and callus formation were observed in the medium supplemented with 1.0 mg/l NAA and 0. 1 mg/l BA, or with 1.0 mg/l 2, 4-D and 0.1 mg/l BA. Callus formation was accelerated by reducing the concentration of mannitol. Calli derived from mesophyll protoplasts and induced in the medium supplemented with 1.0 mg/l 2,

4-D and 0.1 mg/l BA differentiated some adventitious buds in the medium supplemented with 0.035 mg/l GA and 1.0 mg/l BA. Adventitious buds and roots developed in MS medium without plant growth regulator and regenerated plants were successfully transferred to the soil. They showed morphological variations, in the number of stalks.

Acknowledgement

This work was supported in part by a Grant in Aid for Scientific Research (No. 61440010) from Ministry of Education, Science and Culture, Japan.

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