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Plant Regeneration from Mesophyll Protoplasts *Spinacia oleracea* L.

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Mesophyll protoplasts of *Spinacia oleracea* L. (cv. JIROMARU) were isolated from the *in vitro*-grown young seedlings by treatment of the enzyme solution containing 0.1% Pectolyase Y-23, 0.3% Cellulase ONOZUKA RS, CPWsalts and 0.5M mannitol (pH5.8). The most efficient cell division and colony formation occurred in a modified 1/2 MS medium containing 5mg/*l* BA and 1mg/*l* 2, 4-D. Shoots were induced in the following four treatments: a combination of the callus formation medium containing 1mg/*l* BA and 10mg/*l* NAA with the 2 kinds of shoot formation media containing 1mg/*l* kinetin or 5mg/*l* zeatin; a combination of the callus formation medium containing 5mg/*l* BA and 5mg/*l* NAA with the shoot formation medium containing 5mg/*l* BA; a combination of the callus formation medium containing 5mg/*l* BA and 10mg/*l* NAA with the shoot formation medium containing 1mg/*l* kinetin. Regenerated shoots developed roots in the MS liquid medium containing 1mg/*l* IAA or IBA.

Key words: protoplast, culture, plant regeneration, *Spinacia oleracea* L.

Introduction

Spinach is a very important leafy vegetable as a fresh or processed food. Recently, there has been progress in unconventional breeding methods such as somatic hybridization and direct gene transfer. These methods will be very useful for introducing specific traits, such as resistance to heat, disease and discoloration by thermal processing into already established cultivars.

Plant regeneration from tissue culture[1,2] and shoot primordia culture[3] have been accomplished in spinach but there has been no report of success in plant regeneration from protoplasts. The objective of the present study was to investigate the effects of culture media on cell division, callus proliferation and plant regeneration from spinach mesophyll protoplasts.

Materials and Methods

Seeds of *Spinacia oleracea* L. (cv. JIROMARU) used in this examination were purchased from a commercial market. After removing the pericarp, seeds were surface-sterilized with 75% ethanol for 30 sec and sodium hypochlorite solution (5% available chlorine) with a drop of Tween 20 for 15 min, followed by several rinses with sterile distilled water. The seeds were inoculated on the MS medium (Murashige & Skoog 1962)[4] containing 2% sucrose and 8g/*l* agar at pH5.8. Germinated seedlings were grown at 25 ± 1℃ under fluorescent light at 2,000lux, 16hr/day. After about 10 days, aseptic shoots with young leaves (5-6cm in length) were cut from the seedlings and immersed in 0.5M mannitol solution for 15 min without cutting into small pieces. Then

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Table 1. Effect of auxin and cytokinin on the protoplast division and the cell colony formation in mesophyll protoplast culture of spinach

Growth regulator (mg/l)		Cell[1] division	Colony[2] formation	Growth regulator (mg/l)		Cell[1] division	Colony[2] formation
BA 1	NAA 1	−	−	kin. 1	NAA 1	−	−
1	2.5	−	−	1	2.5	−	−
1	5	−	−	1	5	−	−
2.5	1	−	−	2.5	1	−	−
2.5	2.5	−	−	2.5	2.5	−	−
2.5	5	−	−	2.5	5	−	−
5	1	−	−	5	1	−	−
5	2.5	−	−	5	2.5	−	−
5	5	−	−	5	5	−	−
BA 1	2, 4-D 1	+	±	kin. 1	2, 4-D 1	−	−
1	2.5	+	±	1	2.5	−	−
1	5	+	−	1	5	−	−
2.5	1	+	±	2.5	1	−	−
2.5	2.5	+	±	2.5	2.5	−	−
2.5	5	+	±	2.5	5	−	−
5	1	+	+++	5	1	−	−
5	2.5	+	±	5	2.5	−	−
5	5	+	−	5	5	−	−
BA 1	IAA 1	−	−	kin. 1	IAA 1	−	−
1	2.5	−	−	1	2.5	−	−
1	5	−	−	1	5	−	−
2.5	1	−	−	2.5	1	−	−
2.5	2.5	−	−	2.5	2.5	−	−
2.5	5	−	−	2.5	5	−	−
5	1	−	−	5	1	−	−
5	2.5	−	−	5	2.5	−	−
5	5	−	−	5	5	−	−

Composition of basal medium was 1/2 MS salts, MS vitamins, 2% sucrose, and 0.5 M mannitol (pH 5.8).

[1] + : cell division observed, − : no cell division observed (10 days after culture).

[2] Number of colonies per plate (4 weeks after culture).
− : 0, ± : 0～10, ++ : 20～30, +++ : 30 <.

they were incubated in the enzyme solution prepared by adding 0.1% Pectolyase Y-23 and 0.3% Cellulase ONOZUKA RS to CPWsalts[5] solution containing 0.5M mannitol at pH5.8. The enzyme treatment was continued for 4hrs with shaking at 60 strokes/min on a reciprocal shaker at room temperature. After incubation, the enzyme solution was removed, and the leaves were crushed with forceps to isolate protoplasts in a 21% sucrose solution containing CPWsalts. The suspension was filtered through a nylon sieve with 60 μm pore size and centrifuged at 100×g for 5 min. Intact protoplasts floated on surface of the lipuid were collected with a pipette, and suspended in CPWsalts solution containing 0.5 M mannitol, and centrifuged at 100×g for 4 min. This wash sequence was replicated twice.

Table 2. Effect of auxin and cytokinin on the callus formation from protoplast-derived colonies of spinach after one month of culture

Cytokinin (mg/*l*)	Auxin (mg/*l*)	Number of calli/Plate[1)]	
		Plate 1	Plate 2
BA 1	NAA 1	155 SG	0
1	5	159 LG	0
1	10	65 LG	42 LG
5	1	150 SG	47 LG
5	5	145 SW	21 LG
5	10	136 SW	14 LG
10	1	160 SW	0
10	5	169 SW	0
10	10	226 SW	0
BA 1	2, 4-D 1	130 SG	18 SG
1	5	73 SG	0
1	10	50 SG	0
5	1	100 SG	47 SG
5	5	63 SG	12 SG
5	10	16 SG	9 SG
10	1	133 SG	0
10	5	83 SG	0
10	10	75 SG	0
BA 1	IAA 1	137 SG	100 SG
1	5	76 SG	8 SG
1	10	103 SG	0
5	1	192 SG	33 LG
5	5	88 SG	15 LG
5	10	39 LG	0
10	1	181 SG	76 LG
10	5	103 SG	0
10	10	0	0

Protoplasts were cultured in the medium containing 1/2 MS salts, 8P vitamins, 2% sucrose, 0.5 M mannitol, 5 mg/*l* BA and 1 mg/*l* 2, 4-D (pH 5.8).

For the callus formation, basal medium containing 1/2 MS salts and 8P vitamins was used.

[1)] SG : small (<1 mm) and green callus, SW : small and white callus, LG : large (≧2 mm) and green callus.

Plate 1 : Vigorously growing colonies were used; Plate 2 : Weakly growing colonies were used.

The protoplasts were suspended in the protoplast culture media at a density of 1 $\times 10^5$ protoplasts/m*l* in 60 mm ϕ plastic petri-dish. They were cultured at 25℃ under dim light for a week, and were transferred under fluorescent light at 2,000 lux, 16hr/day until plant regeneration. Protoplast culture media were composed of 1/2 MS inorganic salts, MS or 8P vitamins, 2% sucrose and 0.5M mannitol, and were supplemented with various kinds and amounts of auxins and cytokinins as

Table 3. Effect of cytokinin on the shoot formation from protoplast-derived calli of spinach

Callus formation medium[1]		Shoot formation medium					
		BA (mg/*l*)		kinetin (mg/*l*)		zeatin (mg/*l*)	
Cytokinin	Auxin (mg/*l*)	1.0	5.0	1.0	5.0	1.0	5.0
BA 1	NAA 1	−[2]	−	−	−	−	−
1	5	+	+	−	−	+	+
1	10	+	+	+ (2/7)[3]	+	+	+ (2/7)
5	1	+	+	+	+	+	+
5	5	+	+ (1/5)	+	+	+	+
5	10	+	+	+ (1/3)	+	+	+
10	1	*	*	*	*	*	+
10	5	*	*	*	*	*	+
10	10	*	*	*	*	*	+
BA 1	2, 4-D 1	+	+	+	+	+	+
1	5	*	*	*	*	*	*
1	10	*	*	*	*	*	*
5	1	+	+	+	+	+	+
5	5	*	*	*	*	*	*
5	10	*	*	*	*	*	*
10	1	*	*	*	*	*	*
10	5	*	*	*	*	*	*
10	10	*	*	*	*	*	*
BA 1	IAA 1	+	+	−	+	+	−
1	5	+	+	+	+	+	+
1	10	+	+	+	+	+	+
5	1	+	+	+	+	+	+
5	5	+	+	+	+	+	+
5	10	+	+	+	+	+	+
10	1	+	+	+	+	+	+
10	5	−	+	−	−	+	−
10	10	*	*	*	*	*	+

Protoplasts were cultured in the medium containing 1/2 MS salts, 8P vitamins, 2% sucrose, 0.5 M mannitol, 5 mg/*l* 2, 4-D (pH 5.8). Calli obtained from the plate 1 in Table 2 were used for culture materials. MS basal medium was used for shoot formation.

[1] Culture medium was same as Table 2.

[2] − : callus tissues showed browning, + : callus tissues showed growth.

[3] (Number of calli forming shoots/Number of calli transferred)

* : not examined

shown in **Table 1**. Frequencies of cell division and colony formation were much higher in a medium containing 8P vitamins than MS vitamins (data not shown). So we used mainly 8P vitamins in protoplast culture media instead of MS vitamins.

After one month, small colonies were transferred onto callus formation media. These media were composed of 1/2 MS salts, 8P vitamins and 2% sucrose and were supplemented with various kinds and amounts of auxins and cytokinins as shown in **Table 2**.

After about one month in culture, small calli with a diameter of 2mm were transferred onto MS agar medium containing 2% sucrose and one of the cytokinins shown in **Table 3**.

Regenerated shoots were transferred into MS liquid medium containing 2% sucrose and 1.0mg/*l* IAA or IBA to stimulate root development.

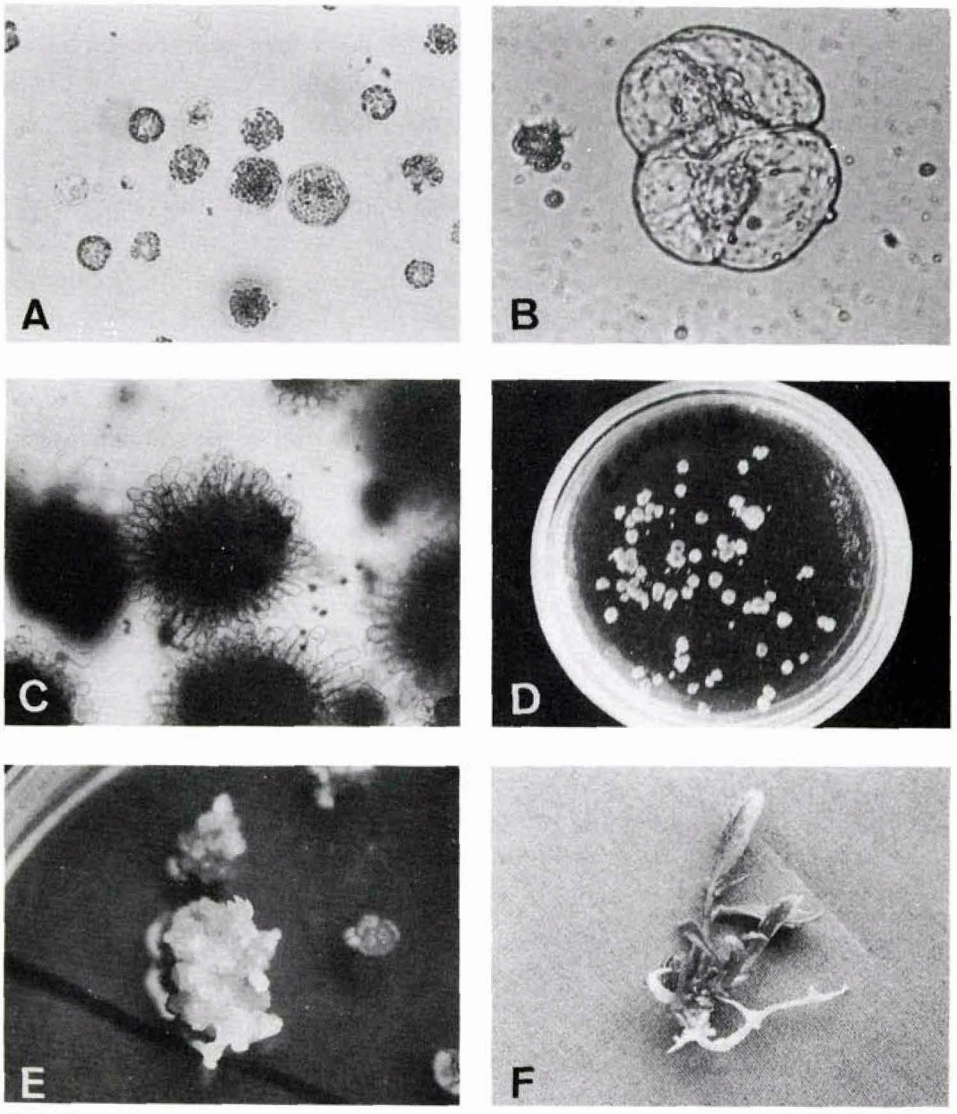

Fig. 1. (A) Freshly isolated mesophyll protoplasts from *in vitro*-grown young seedlings of *Spinacia oleracea*.
(B) The first cell division occurred one week after protoplasts isolation.
(C) Cell colonies obtained from protoplasts about one month after protoplasts isolation.
(D) Protoplast-derived calli (2 months after protoplasts isolation).
(E) Adventitious shoots regenerated on 1/2 MS shoot formation medium (about 3 months after protoplasts isolaion).
(F) Complete plant obtained in root regeneration medium (about 4 months after protoplasts isolation).

Results and Discussion

In order to isolate protoplasts from plant mesophyll tissues, plants are often cut into small pieces to achieve more effective enzyme treatment. However, in the case of spinach, only a few protoplasts could be isolated from mesophyll tissues when this method was used, and most of them were broken during the isolation process. The color of the enzyme solution changed to darkgreen after treatment. We considered that the color change was caused by a large amount of cell sap eluted from protoplasts by degradation. Therefore, we treated intact plant leaves with enzyme solution using the methods described in the section of materials and methods. As a result, many intact and viable protoplasts could be obtained in this study (**Fig. 1-A**).

Protoplasts elongated within 3-4 days in

all the media shown in **Table 1** but cell division did not occur and protoplasts broke down during culture in the media containing no 2, 4-D. First cell division occurred within one week in the medium containing both BA (1-5mg/*l*) and 2,4-D (1-5mg/*l*) (**Fig. 1-B**). Especially in the medium containing 5mg/*l* BA and 1mg/*l* 2, 4-D, cell division was vigorous and a large number of colonies were formed (**Fig. 1-C**).

After about one month of culture, the colonies obtained in this medium were transferred onto the callus formation medium shown in **Table 2**. The growth and the number of colonies differed among the petri-dishes, so the colonies were classified into two types according to the growth level (vigorous and weak).

After about one month of culture, many calli grew well in various media except one medium containing 10mg/*l* BA and 10 mg/*l* IAA (**Fig. 1-D**). The number of calli that grew well from the colonies of weak growth were fewer than those from the colonies of vigorous growth. These results suggest that the condition or the growth level of the transferred colonies should have a great influence on the subsequent callus proliferation.

Calli which grew to 2mm in diameter were transferred onto the regeneration media supplemented with kinetin, BA or zeatin. Most of them grew further, and vigorous callus growth was observed especially in the medium supplemented with zeatin. After 40 days of culture, shoots were induced in the following four treatments : a combination of the callus formation medium containing 1mg/*l* BA and 10 mg/*l* NAA with the 2 kinds of shoot formation media containing 1mg/*l* kinetin or 5 mg/*l* zeatin ; a combination of the callus formation medium containing 5mg/*l* BA and 5mg/*l* NAA with the shoot formation medium containing 5mg/*l* BA ; a combination of the callus formation medium containing 5mg/*l* BA and 10mg/*l* NAA with the shoot formation medium containing 1mg/*l* kinetin (**Fig. 1-E**).

In order to induce adventitious roots, regenerated shoots were transferred into fresh MS liquid medium supplemented with 1mg/*l* IAA or IBA, and cultured with shaking 100rpm by rotary shaker. After about one month of culture, white roots were formed from the shoots (**Fig. 1-F**).

In the present study, we have achieved a plant regeneration system for *Spinacia oleracea* L., using the intact and viable protoplasts obtained from intact leaves without cutting them into small pieces. Further studies are needed to establish a procedure of more efficient regeneration of plants from protoplast-derived calli.

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