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An Improved Procedure for Protoplast Culture and Plant Regeneration of Spinach (*Spinacia oleracea* L.)

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Protoplasts of *Spinacia oleracea* L. were cultured in a modified half-strength MS medium supplemented with KM8p vitamins, 1.0 mg·liter^{-1} BA, 1.0 mg·liter^{-1} 2,4-D, and glucose or mannitol. Plating efficiency in the medium containing glucose as an osmoticum was much higher than that in the medium containing mannitol. The addition of KM8p organic acids to the glucose medium promoted the division of protoplasts slightly; the same addition to a medium containing mannitol appeared inhibitory to the cell division. Several shoots were formed from calli obtained on the MS medium containing 1.0–10.0 mg·liter^{-1} IAA or IBA. Adventitious root was developed most vigorously on the MS medium containing 2% sucrose, 0.5 mg·liter^{-1} IBA, and 0.8% agar when pH was adjusted to 6.3. Some regenerated plantlets transferred to a greenhouse produced many seeds.

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

Introduction

Spinach, *Spinacia oleracea* L., belongs to Chenopodiaceae and is a very important vegetable, because it contains many vitamins and minerals, such as carotene, vitamin C, calcium, and iron. However, spinach does not generally tolerate high temperatures and acid soil, and may contain a large amount of oxalate which interferes with Ca absorption if excessive amounts are ingested. Protoplast culture has become widely applied to studies on cell biology and molecular biology, because of its usefulness in genetic transformation, somatic hybridization, and induction of somaclonal variation.

We recently reported the first successful plant regeneration form mesophyll protoplasts of spinach (Goto and Miyazaki, 1992). However, efficiency of shoot regeneration was very low and only a few plantlets were obtained. Fujita et al. (1994) reported that callus formed from mesophyll protoplasts of spinach, but plantlets could not be regenerated from the protoplasts.

In the present study. we report a more efficient procedure for the protoplasts division and plant regeneration from mesophyll protoplasts of spinach.

Materials and Methods

Mesophyll protoplasts of *Spinacia oleracea* L. cv. Jiromaru were isolated as described previously (Goto and Miyazaki, 1992).

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Protoplasts (1×10^5 protoplasts·ml^{-1}) were cultured in 60×15 mm plastic Petri dishes containing 3 ml of liquid medium and the dishes were sealed with Parafilm (American National Can). The liquid medium contained a half-strength of MS inorganic salts (Murashige and Skoog, 1962), except NH_4NO_3 (200 mg·$liter^{-1}$), KM8p vitamin (Kao and Michayluk, 1975), 1.0 mg·$liter^{-1}$ BA, 1.0 mg·$liter^{-1}$ 2,4-D and various combinations of glucose and mannitol with or without KM8p organic acids (20 mg·$liter^{-1}$ sodium pyruvate, 40 mg·$liter^{-1}$ citric acid, 40 mg·$liter^{-1}$ malic acid and 40 mg·$liter^{-1}$ fumaric acid). The pH of the culture medium was adjusted to 5.8. The cultures were maintained at 25℃ in the dark for a week, and then placed under a 16-hr photoperiod. The light (2,000 lx) was supplied by fluorescent lamps. Plating efficiency (PE) was defined as the percentage of deviding protoplasts after 10 days of culture against originally plated protoplasts.

After a month of culture, protoplast-derived micro-colonies were transferred onto callus formation media. These media were composed of a half strength MS inorganic salts, KM8p vitamins, 0.1 mg·$liter^{-1}$ BA, 1.0 mg·$liter^{-1}$ GA_3, 2% sucrose, 0.25% gellan gum (Wako Pure Chemical Industries Ltd.) and various concentraions of auxin (IAA, IBA, NAA or 2,4-D). About one month later, the calli with a diameter of about 2 mm were transferred onto MS medium containing 2% sucrose, 0.25% gellan gum without plant growth regulators for shoot regeneration. All media for callus formation and shoot regeneration were adjusted to pH 5.8.

Regenerated shoots were detached from the callus and transferred for rooting to test tubes containing MS media supplemented with various concentrations of IBA and sucrose, and 0.25% gellan gum or 0.8% agar (Wako Pure Chemical Industries Ltd.), and adjusted to three values of pH. These test tubes were sealed with millipore films to prevent vitrification and maintained at 25 ℃ and a 10-hr photoperiod.

Regenerated plantlets were transferred to individual pots containing vermiculite and covered with a small plastic bag to

Table 1. Effect of sugars, sugar alcohol, and KM8p organic acids (KM8pOA) on the plating efficiency of spinach protoplasts.

Composition of medium	Plating efficiency (%)
2% sucrose + 0.5M mannitol	3.3±0.3
2% glucose + 0.5M mannitol	5.7±0.5
1% sucrose + 1% glucose + 0.5M mannitol	3.5±0.1
2% sucrose + 0.5M mannitol + KM8pOA	0
2% glucose + 0.5M mannitol + KM8pOA	0
0.5M glucose	9.9±0.5
2% sucrose + 0.5M glucose	8.9±0.2
0.5M glucose + KM8pOA	9.9±0.3
2% sucrose + 0.5M glucose + KM8pOA	8.5±0.1

Each value represents the mean ± SE after 10 days of culture.

maintain high humidity for 1-2 weeks. Subsequently, the plants were grown in a greenhouse.

Results

Protoplasts began to divide after 4-5 days of culture. Plating efficiency of the medium containing glucose as an osmoticum/carbon source was much higher than that of the medium containing mannitol (Table 1). The highest plating efficiency (9.9%) was obtained from the

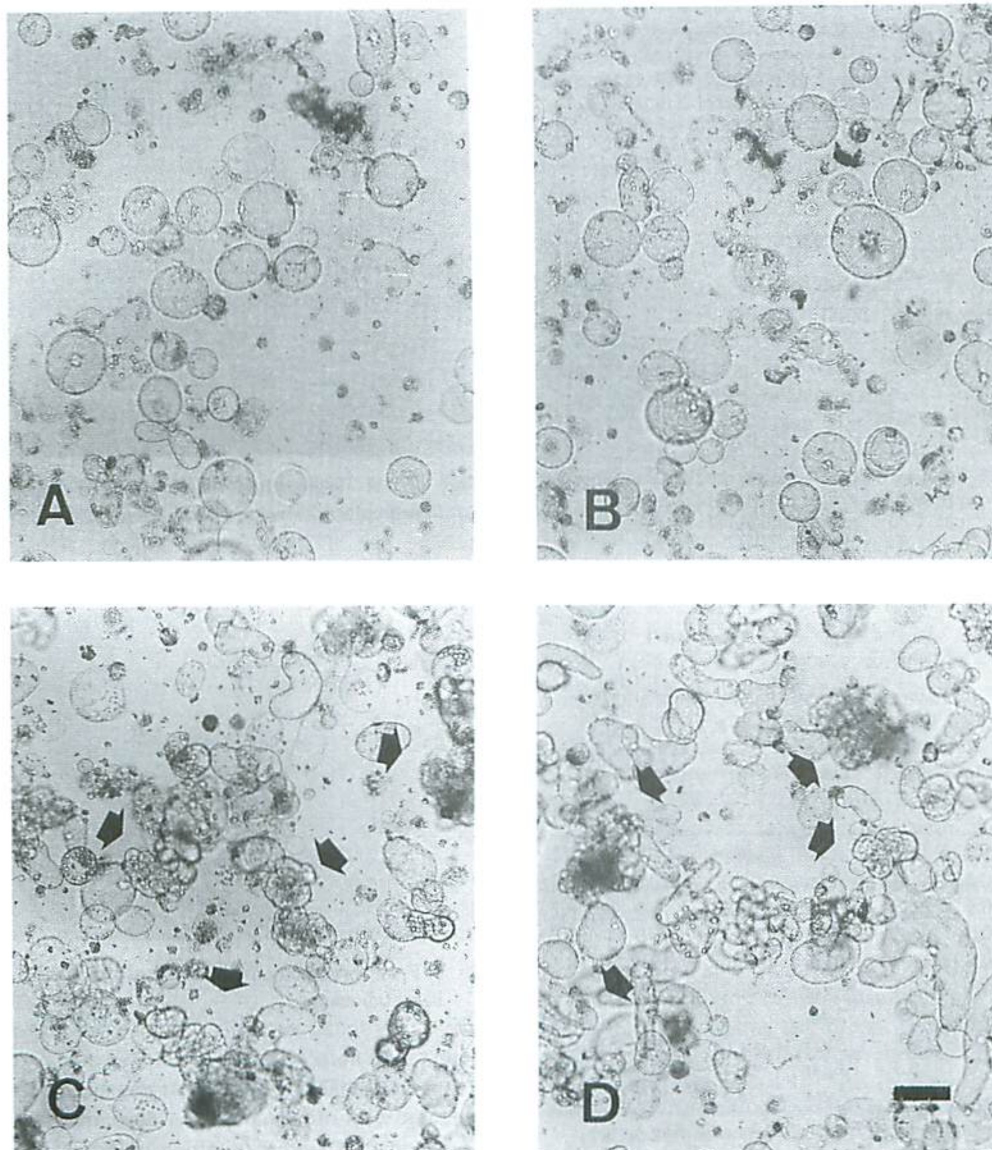

Fig. 1. Photomicrographs of protoplasts dividing on media containing mannitol and sugars in various combination with KM8p organic acids.
A: 0.5M mannitol and 2% sucrose. B: 0.5M mannitol and 2% glucose. C: 0.5M glucose. D: 0.5M glucose and 2% sucrose. Bar=100 μm. Arrows indicate colonies.

medium containing 0.5M glucose.

In the medium containing both KM8p organic acid and 0.5M mannitol, most of the protoplasts were damaged and no cell division was observed (Table 1, Fig. 1A and B). On the contrary, many protoplasts divided in the medium containing 0.5M glucose with or without KM8p organic acids, and formed a large number of micro-colonies (Fig. 1C and D). Protoplast division proceeded more rapidly in the medium containing KM8p organic acids.

Protoplast-derived micro-colonies developed more vigorously on the callus formation media containing 1.0-10.0 $mg \cdot liter^{-1}$ IAA or 1.0-5.0 $mg \cdot liter^{-1}$ IBA one month after transfer (Table 2). Callus turned brown on the medium containing 10.0 $mg \cdot liter^{-1}$ IAA or 5.0 $mg \cdot liter^{-1}$ IBA. Compact and green calli were formed on the medium with 5.0 $mg \cdot liter^{-1}$ IAA or 1.0 $mg \cdot liter^{-1}$ IBA. About one month after transfer to shoot regeneration medium, green shoot primordia appeared and some of the calli formed embryos. Several shoots were regenerated from calli developed on the callus formation media containing 1.0-10.0 $mg \cdot liter^{-1}$ IAA or IBA. Calli derived from the media containing NAA or 2,4-D did not regenerate shoots, whereas that containing 5.0 $mg \cdot liter^{-1}$ 2,4-D did.

Adventitious roots were formed from the shoots excised from the calli about one month after transfer to a rooting medium. Among the three media with different pH, adventitious roots were developed most vigorously on the medium adjusted to pH 6.3 (Table 3). Adventitious root formation and development were hindered in the medium adjusted to pH 6.8.

On the medium containing agar as a

Table 2. Effect of auxin on callus formation medium on shoot regeneration after a month culture.

Concn. of auxin[z] ($mg \cdot liter^{-1}$)		No. of calli transplanted[y]	No. of calli forming shoot	Shoot formation ratio(%)
IAA	1	22	1	4.5
IAA	5	36	5	13.4
IAA	10	28	3	10.7
IBA	1	31	3	9.7
IBA	5	30	2	6.7
IBA	10	8	1	12.5
NAA	1	13	0	0
NAA	5	19	0	0
NAA	10	13	0	0
2,4-D	1	24	0	0
2,4-D	5	6	1	16.7
2,4-D	10	12	0	0

[z] Colonies were cultured on 1/2 MS medium contained KM8p vitamins, 0.1 $mg \cdot liter^{-1}$ BA, 1.0 $mg \cdot liter^{-1}$ GA_3, 2.0% sucrose, 0.25% gellan gum, and several kinds and concentrations of auxins.

[y] Calli developed more than 2 mm in diameter were transplanted to MS medium containing 2.0% sucrose, 0.25% gellan gum.

Table 3. Frequency of adventitious root formation from shoots derived from protoplasts.[z]

IBA ($mg \cdot liter^{-1}$)	Sucrose (%)	pH	Gelling agent	Adventitious root (%)[y]		
				< 2 cm	≥ 2 cm	total
0.5	0.5	5.8	Agar	40.0	6.7	46.7
0.5	0.5	6.3	Agar	26.7	20.0	46.7
0.5	0.5	6.8	Agar	26.7	0	26.7
0.5	0.5	6.3	Gellan gum	26.7	0	26.7
0	0.5	6.3	Agar	20.0	13.3	33.3
0.1	0.5	6.3	Agar	26.7	6.7	33.3
1.0	0.5	6.3	Agar	26.7	13.3	40.0
0.5	0	6.3	Agar	0	6.7	6.7
0.5	1.0	6.3	Agar	26.7	20.0	46.7
0.5	2.0	6.3	Agar	13.3	40.0	53.3

[z] Shoots were induced from callus by culturing on the callus formation medium (1/2 MS medium contained KM8p vitamins, 0.1 $mg \cdot liter^{-1}$ GA_3) for about one month and transferred to shoot formation medium (MS medium without plant growth regulators).

[y] 15 shoots were transplanted in each experiment.

gelling agent, numerous, large adventitious roots grew vigorously, more so than did the roots on the medium containing gellan gum. Adventitious root formation and their development were promoted by IBA; the maximum was attained in the medium containing 0.5 mg·liter^{-1} IBA.

The concentration of sucrose also affected the formation of adventitious roots. When the shoots were transplanted to the medium lacking sucrose, most did not produce adventitious roots but died. Mutoh (1991) reported that the development of adventitious root was inhibited by sucrose. In our study, adventitious roots developed most vigorously on the medium containing 2% sucrose.

When regenerated plantlets were transplanted to the pot and grown in a greenhouse (Fig. 2), the acclimatized plants flowered within 2 months after transplanting. Their floral characteristics revealed that they were dioecious male, dioecious female or monoecious. The monoecious plants produced many seeds by self-pollination (Fig. 3).

Fig. 2. A regenerated plant from a mesophyll protoplast-derived callus. Bar=1.0 cm.

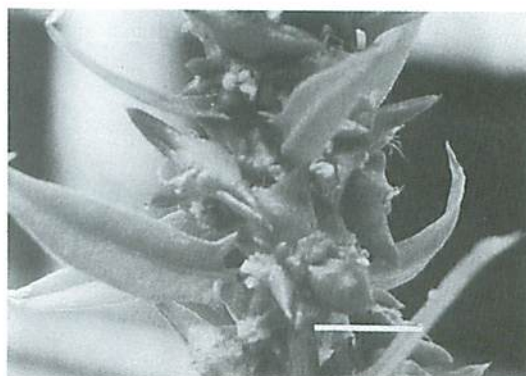

Fig. 3. Fruit formation on a monoecious regenerant. Bar=0.5 cm.

Discussion

In our previous study (Goto and Miyazaki, 1992), we used protoplast culture medium containing mannitol as an osmoticum. However, in this study, we found that glucose used for both carbon source and osmoticum was superior to mannitol. The same result was reported by Mii et al. (1991).

KM8p medium often used for protoplast culture contains much more kinds of bitamins and organic substances than the other media. We reported that frequencies of protoplast division and colony formation were much higher in the medium containing KM8p vitamins than MS vitamins (Goto and Miyazaki, 1992). Protoplasts were injured by KM8p organic acids when they were cultured on the medium containing 0.5M mannitol, whereas they were not in the medium containing 0.5M glucose. Furthermore, they divied more rapidly in the presence of KM8p organic acids than in the medium without them.

In spinach, shoot regeneration from

the callus developed from a hypocotyl segment was achieved in the medium containing IAA and GA_3 (Sasaki, 1989; Satoh et al., 1992). Xiao et al. (1993) reported that somatic embryos were obtained from the calli which were induced on solid media supplemented with IAA and GA_3 and then subcultured on IAA containing media. In the present study, shoot regeneration was observed in the medium containing 1.0 mg•liter^{-1} GA_3 and either 5.0-10.0 mg•liter^{-1} IAA or 1.0-5.0 mg•liter^{-1} IBA. These media were more effective for inducing shoot regeneration from the protoplast-derived calli than previous ones employed by us. Induction of shoot from the callus could be observed only in the media containing GA_3 (Data was not shown). However, the rate of shoot regeneration was lower than that obtained in the other tissue culture studies (Sasaki, 1989; Al-Khayri et al., 1991; Satoh et al., 1992; Xiano et al., 1993; Okuse et al., 1994). Thus, it is necessary to develop a more effective medium for shoot regeneration from protoplast-derived calli.

Although the pH of medium is usually adjusted to pH 5-6, adventitious root formation and subsequent root growth were more vigorous in the medium adjusted to pH 6.3 than in pH 5.8. Spinach is very sensitive to soil acidity; it grows poorly in soils below pH 5.5 (Zimmerley, 1924). Seemingly, this character influences both the formation and development of adventitious roots.

Fujita et al. (1994) isolated spinach protoplasts and induced callus formation but they were unable to regenerate plantlets. In this study, an efficient procedure was developed by modifying the culture medium. This procedure could be applied to new methods, such as cell fusion and direct gene transfer in spinach breeding.

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