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Stimulation of Protoplast Division of Spinach (*Spinacia oleracea* L.) by the Addition of Citric Acid

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The effects of organic acids on protoplast culture in spinach were investigated. Protoplasts obtained from young leaves were cultured in a modified 1/2 Murashige and Skoog's inorganic medium, containing KM8p vitamins, 1.0 mg·liter^{-1} BA, 1.0 mg·liter^{-1} 2,4-D, and 0.5 M mannitol or 0.5 M glucose supplemented with sodium pyruvate, citric acid. malic acid, fumaric acid, succinic acid, maleic acid, malonic acid, or several combinations thereof. When 0.5 M glucose was present in the medium as an osmoticum, the organic acids (except for malonic acid) stimulated protoplast division, but when 0.5 M mannitol was present instead of glucose, they inhibited the division of protoplasts. Citric acid was most effective in protoplast division, the highest frequency being obtained in the medium supplemented with 0.1 mM citric acid and 0.5 M glucose. The growth of the colony followed a trend similar to that of protoplast division, yielding numerous micro-calli. Citric acid in the protoplast culture medium also induced maximum shoot regeneration. Division of protoplasts obtained from leaves of different plant ages was enhanced by citric acid; a high frequency of cell division was maintained in seedlings up to 25-day-old. The addition of citric acid to the medium promoted protoplasts division in the density range of 3.0 to 20×10^4 protoplasts·ml^{-1}, but it was more effective at lower plating densities. Therefore, our results confirm the usefulness of citric acid for spinach protoplast culture.

Key words : protoplast, cell division, citric acid, osmoticum, *Spinacia oleracea* L.

Introduction

Spinach, which originated from Middle East Asia is one of the most important vegetables in the world being cultivated widely in the temperate regions, subtropical and tropical highlands. Spinach is rich in carotene, ascorbic acid, riboflavin, iron, and calcium. In addition, recent studies have shown that spinach inhibits melanin production (Baba et al., 1996), suppresses the growth of cancer cells (Tsushida and Shinohara, 1997), and possesses superoxide anion radical-scavenging ability (Nishibori and Namiki, 1998). However, spinach is sensitive to soil acidity and hot weather; conversely it thrives well in relatively cool weather and tolerates frost better than do most vegetable crops.

Genetic manipulations based on a protoplast culture system may be advantageous for the direct introduction of resistant genes from other species by cell fusion or electropolation. A prerequisite for the use of such techniques is a reliable plant regenera-

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tion system. Although a number of studies on protoplast culture of spinach have been reported (Nakagawa et al., 1985; Goto and Miyazaki, 1992; Komai et al., 1996; Goto et al., 1996, 1998), a more detailed study is needed to apply protoplast culture to the genetic manipulations because of its low plating efficiency. When various combinations of osmoticum and organic acids in the media for spinach protoplast culture were tested, protoplast division was found to be significantly affected by osmoticum and KM8p (Kao and Michayluk, 1975) organic acids (Goto et al., 1996). Very little is known about the effects of organic acids on protoplast division, although they are present in the KM8p medium. To establish a more reliable protoplast culture system for spinach, the optimum kind and concentrations of organic acids are described in this paper.

Materials and Methods

Protoplasts were isolated from 10-day-old seedlings (cv. Jiromaru), as described previously (Goto and Miyazaki, 1992) and cultured on modified 1/2 Murashige and Skoog's (Murashige and Skoog, 1962) inorganic medium (MS) except NH_4NO_3 (200 mg·liter^{-1}) containing KM8p vitamins, 1.0 mg·liter^{-1} BA, and 1.0 mg·liter^{-1} 2,4-D. Preliminary examinations revealed that a medium with an osmoticum pressure of 0.5 M glucose yielded the best. Hence, protoplasts were cultured on a basal medium containing 0.5 M mannitol or 0.5 M glucose, and supplemented with 20 mg·liter^{-1} (0.18 mM) sodium pyruvate, 40 mg·liter^{-1} (0.21 mM) citric acid, 40 mg·liter^{-1} (0.30 mM) malic acid, 40 mg·liter^{-1} (0.34 mM) fumaric acid, 40 mg·liter^{-1} (0.34 mM) succinic acid, 40 mg·liter^{-1} (0.34 mM) maleic acid, 40 mg·liter^{-1} (0.38 mM) malonic acid, or several combinations thereof (**Table 1**). Protoplasts were prepared at a density of 1.0×10^5 protoplasts·ml^{-1} in a 35 × 10 mm plastic Petri-dish containing 1.0 ml of culture medium. A 0.25-ml aliquot of same fresh media was added at 10-day intervals.

After a month of culture, the micro-calli derived by several combinations of organic acids (**Table 2**) were transferred onto 1/2 MS inorganic medium containing KM8p vitamins, 0.1 mg·liter^{-1} BA, 1.0 mg·liter^{-1} IBA, 1.0 mg·liter^{-1} GA_3, 2.0% sucrose, and 0.25% gellan gum. About a month later, calli

Table 1 Effect of organic acids and osmoticum on the frequency of cell division (%) in spinach protoplasts[z].

Organic acid	Osmoticum	
	Mannitol	Glucose
None (Control)	1.81a[y]	4.76c
Sodium pyruvate (P)	1.11bc	6.40b
Citric acid (C)	0.40ef	9.27a
Malic acid (M)	0.68de	6.79b
Fumaric acids (F)	1.04bcd	5.89bc
C+M+F	0.50ef	5.34bc
P+M+F	0.32ef	6.26b
P+C+F	0.22f	6.12bc
P+C+M	0.11f	5.37bc
P+C+M+F	0.12f	5.86bc
Succinic acid	0.36ef	6.41b
Maleic acid	1.12b	5.79bc
Malonic acid	0.29ef	3.01d

[z] The frequency of cell division defined as the percentage of diving protoplasts over those originally plated after 10 days of culture.

[y] Different letters within the column show significant difference by Duncan's new multiple range test at $P=0.05$. Data were expressed as the average of at least 3 independent experiments.

Table 2 Effect of organic acids in the protoplast culture medium (0.5 M glucose osmoticum) on the shoot regeneration ratio from the protoplast-derived callus[z].

Organic acids contanined in protoplast culture media	Shoot formation ratio (%)
None (Control)	7.1bc[y]
Sodium pyruvate (P)	8.2ab
Citric acid (C)	13.0a
Malic acid (M)	2.7c
Fumaric acids (F)	10.0ab
C+M+F	5.9bc
P+M+F	9.4ab
P+C+F	8.6ab
P+C+M	5.8bc
P+C+M+F	5.3bc

[z] Micro-calli were cultured on 1/2 MS medium containing KM8p vitamins, 0.1mg·liter^{-1} BA, 1.0mg·liter^{-1} IBA, 1.0mg·liter^{-1} GA_3, 2.0% sucrose, and 0.25% gellan gum. Calli which developed to about 2.0mm in diameter were transplanted to MS medium containing 2.0% sucrose and 0.25% gellan gum. Data was recorded after one month of culture.

[y] Different letters within the column show significant difference by Duncan's new multiple range test at P=0.05. Data were means of two replicates with about 30 calli per replication.

which developed to about 2.0 mm in diameter were transplanted to MS medium, containing 2.0% sucrose and 0.25% gellan gum, without plant growth regulators to induce the shoot regeneration.

Protoplasts were cultured in the basal medium, containing 0.5 M mannitol or glucose and supplemented with 0, 0.05, 0.10, 0.50, and 1.00 mM citric acid to estimate the relation between varying concentrations of citric acid and protoplast division. Three ml of protoplast suspension were plated in each 60 × 15 mm plastic Petri-dish at a density of 1.0 × 10^5 protoplasts·ml^{-1}.

To investigate the effects of citric acid, protoplasts were isolated from seedlings at 5, 10, 15, 20, 25, or 30 days of age, and cultured in the basal media containing 0.5 M glucose supplemented with or without 0.1 mM citric acid at a density of 1.0 × 10^5 protoplasts·ml^{-1}. Then, protoplasts, isolated from 10-day-old seedlings, were cultured at a density of 1.0 to 20 × 10^4 protoplasts·ml^{-1} on the above medium.

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.

The frequency of cell division defined as the number of dividing cells per initial protoplast, was recorded 10 days after plating. Data represent the mean of at least three replications, using at least two culture dishes.

Results

1. Effect of organic acids

The frequencies of cell division differed, depending on the kinds of organic acid in the medium (Table 1). When glucose was used in the media as an osmoticum, citric acid gave the best result. Five other organic acids and their combinations proved to be moderately more effective than the organic acid-free medium; malonic acid was least effective. When mannitol was used as the osmoticum, protoplasts, exposed to organic acids, divided less frequently than did those in the organic acid-free medium. The inhibition of protoplast division by organic acids, except for sodium pyruvate, fumaric acid, and maleic acid, was significant, particularly their combination.

A similar tendency was observed in the proliferation of cell colonies. Numerous

micro-calli were obtained in the medium containing citric acid and 0.5 M glucose, but the growth rate of calli was not significantly affected by organic acids. Shoot regeneration from calli occurred in all media. (Table 2), but, the maximum number was obtained from calli cultured in the medium containing citric acid. No significant differences were observed in the shoot regeneration ratio among the other organic acids.

2. Effect of citric acid

The above results demonstrate that citric acid enhances division of protoplast when glucose is used as an osmoticum. Therefore, we cultured protoplast in media containing varying concentrations of citric acid to screen a favorable concentration for protoplast division (Table 3). When glucose was used as the osmoticum, the frequency of cell division and colony formation increased with higher citric acid concentrations up to 0.10 mM, but decreased at higher concentrations (Fig. 1 A, B). On the contrary, when mannitol was used as the osmoticum, citric acid adversely affected the division of protoplast (Fig. 1 C, D), the frequencies being lower than that of the control. Exposure of protoplasts to 0.5 to 1.0 mM citric acid deleteriously affected their viability so that protoplast division did not occur.

Table 3 Effect of citric acid concentration and osmoticum in the media on the frequency of cell division (%) in spinach protoplasts[z].

Concentrations of citric acid (mM)	Osmoticum	
	Mannitol	Glucose
0	1.54a[y]	7.05c
0.05	0.46b	8.99b
0.10	0.27c	10.61a
0.50	0.00d	5.28d
1.00	0.00d	1.23e

[z] The frequency of cell division defined as the percentage of diving protoplasts over those originally plated after 10 days of culture.

[y] Different letters within the column show significant difference by Duncan's new multiple range test at P=0.05. Data were expressed as the average of at least 3 independent experiments.

Citric acid affected the frequency of cell division of protoplasts isolated from seedlings different ages (Fig. 2). Maximum frequencies of cell division were obtained from 10-day-old seedlings in media with and without citric acid (12.59% and 8.18%, respectively). Protoplasts from 30-day-old seedlings had a low frequency of cell division; some donor plants developed seed stalks. Citric acid enhanced the protoplast division derived from donor plants of all ages. The frequency of cell division of the protoplasts from seedlings up to 25-day-old was higher in the medium with citric acid than was the maximum frequency in a medium without citric acid.

Protoplasts cultured with citric acid at various plating densities (Fig. 3) revealed that the frequency of cell division increased with increasing density up to $7-10 \times 10^4$ protoplasts$\cdot$ml^{-1}, but decreased at higher densities in the control. Although citric acid in the medium promoted the division of protoplast at all plating densities, high frequencies were obtained at lower plating densities.

Discussion

KM8p organic acids composed of sodium pyruvate, citric acid, malic acid, and fumaric acid are often added to protoplast culture medium as a carbon source or for enhancing tolerance to stress (Komamine et al., 1990).

Protoplast activity and division were stimulated by these organic acids when the osmoticum was glucose, but were inhibited when it was mannitol. Although when the organic acids are added to the medium, glucose is often used as an osmoticum (Kao and Michayluk, 1975; Bladier and Chagvardieff, 1993; Bhadra et al., 1994), Wade et al. (1989) cultured the protoplasts of asparagus in the medium containing 0.7

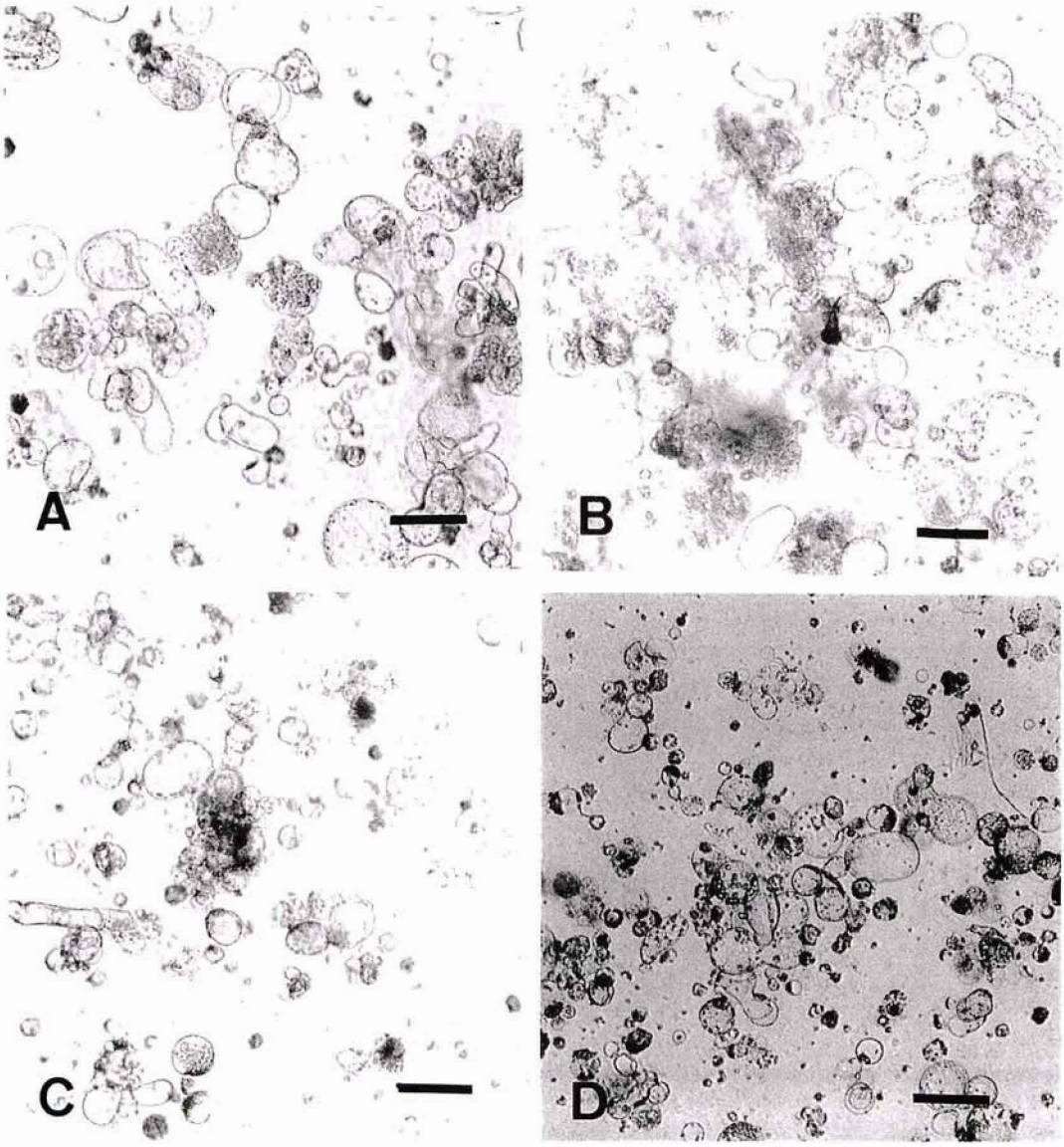

Fig. 1 Protoplasts dividing on the media containing various concentrations of citric acid after 10 days of culture.
The protoplasts were cultured in the media containing glucose supplemented with 0 mM (A) or 0.1 mM (B) citric acid, or mannitol supplemented with 0 mM (C) or 0.1mM (D) citric acid. The bars represent 100 μm.

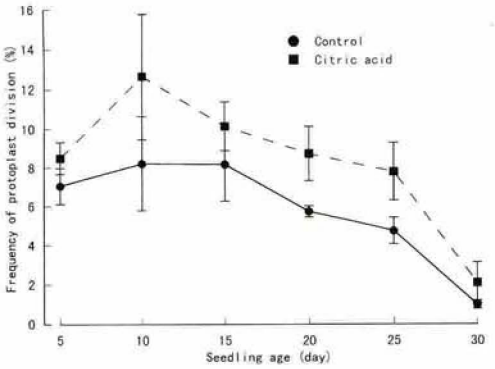

Fig. 2 Effect of seedling age and citric acid on the frequency of cell division in spinach protoplasts.
Protoplasts were cultured in the protoplast culture media containing 0.5 M glucose supplemented with or without 0.1 mM citric acid. The frequency of cell division was defined as the percentage of dividing protoplasts over those originally plated after 10 days of culture. Vertical bars represent SD.

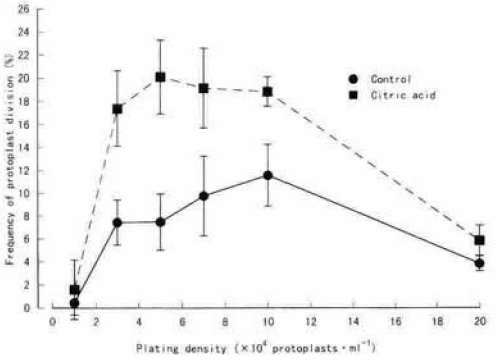

Fig. 3 Effect of plating density and citric acid on the frequency of cell division in spinach protoplasts.
Protoplasts were cultured in the protoplast culture media containing 0.5 M glucose supplemented with or without 0.1 mM citric acid. The frequency of cell division was defined as the percentage of dividing protoplasts over those originally plated after 10 days of culture. Vertical bars represent SD.

M mannitol and KM8p organic acids. The effects of combining organic acids and the osmoticum on the protoplast division are likely to vary by the material.

KM8p organic acids enhanced protoplast division but not the frequency of cell division (Goto et al., 1996). In this study, we found that citric acid stimulated the protoplast division most efficiently, but that its effectiveness decreased when combined with KM8p organic acids. The highest frequency of cell division (10-20 %) was obtained in the medium containing 0.1 mM citric acid and 0.5 M glucose. The calli induced from the protoplasts cultured on the medium also had the highest ratio of shoot regeneration. These results indicate that the addition of citric acid to the protoplast culture medium stimulates protoplast division as well as organogenesis.

Ojima and Ohira (1976) studied the effects of organic acids on the growth of rice suspension culture; they reported that organic acids with a high level of pKa ratio prevent a decrease of cell growth in the medium containing NH_4^+. They proposed that organic acids inhibit the reduction of pH caused by the absorption of NH_4^+ in medium because of the buffering capacity. In this study, protoplast division was enhanced by citric acid, which has a high pKa ratio, whereas malonic acid inhibited division of protoplast in spite of its high pKa ratio which suggest that the stimulation of the division by citric acid may have little relation to its buffer capacity.

The frequencies of cell division increased with the age of donor plants up to 10-to 15-day-old, and then decreased gradually. In protoplasts isolated from 25-day seedlings,

the high frequency of cell division in the medium with citric acid persisted compared with a medium without citric acid. Similar effects of citric acid on protoplast division were observed at different plating densities. Citric acid was beneficial for protoplast division at all plating densities tested, especially at a density of 3 to 10 $\times$ 10^4 protoplasts$\cdot ml^{-1}$.

In conclusion, we found that organic acids promote protoplast division, and that the addition of citric acid alone to the culture medium maximized the process with spinach. Although the mechanism of action of citric acid in enhancing the division and the organogenesis of spinach protoplasts is still unclear, its addition stimulates protoplast division even under adverse conditions. These effects of citric acid stabilized the reproducibility of the protoplast culture, the lack of which yields erratic results. Furthermore, our results should benefit those engaged in protoplast culture of other plant species.

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Literature Cited

Baba, N., H. Shinmoto, M. Kobori and T. Tsushida. 1996. Effects of some edible plants on melanin production, immunoglobulin serection and differentiation of cultured mammalian cell lines. Nippon Shokuhin Kagaku Kogaku Kaishi **43**: 622-628 (In Japanese with English summary).

Bhadra, S. K., N. Hammatt, J. B. Power and M. R. Davey. 1994. A reproducible procedure for plant regeneration from seedling hypocotyl protoplasts of *Vigna sublobata* L. Plant Cell Reports **14**: 175-179.

Bladier, C. and P. Chagvardieff. 1993. Growth and photosynthesis of photoautotrophic callus derived from protoplasts of *Solanum tuberosum* L. Plant Cell Reports **12**: 307-311.

Goto, T. and M. Miyazaki. 1992. Plant regeneration from mesophyll protoplasts of *Spinacia oleracea* L. Plant Tissue Culture Lett. **9**: 15-21.

Goto, T., M. Miyazaki and M. OKu. 1996. An improved procedure for protoplast culture and plant regeneration of spinach (*Spinacia oleracea* L.). J. Japan. Soc. Hort. Sci. **65**: 349-354.

Goto, T., M, Miyazaki and M. Oku. 1998. Varietal variations in plant regeneration from protoplasts in spinach (*Spinacia oleracea* L.). J. Japan. Soc. Hort. Sci. **6 7**: 503-506.

Kao, K. and M. R. Michayluk. 1975. Nutritional requirements for *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta (Berl.) **126**: 105-110.

Komai, F., K. Masuda, T. Harada and I. Okuse. 1996. Plant regeneration from adventitious roots of spinach (*Spinacia oleracea* L.) grown from protoplasts. Plant Science **120**: 89-94.

Komamine, A., K. Ojima, U. Sankawa , K. Shono, H. Harada, K. Hinata, T. Fujimura and H. Yamaguchi (eds). 1990. Shokubutsu biotechnology Jiten. p.78. Asakura Shoten, Tokyo. (In Japanese).

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

Nakagawa, H., H. Tanaka, T. Oba, N. Ogawa and M. Iizuka. 1985. Callus formation from protoplasts of cultured *Spinacia oleracea* cells. Plant Cell Reports **4**: 148-150.

Nishibori, S. and K. Namiki. 1998. Superoxide anion radical-scavenging ability of fresh and heated vegetable juice. Nippon Shokuhin Kagaku Kogaku Kaishi **45**: 144-148 (In Japanese with English summary).

Ojima, K. and K. Ohira. 1976. The growth of rice suspension culture cells by nitrate and ammonium nitrogen. Jpn. J. Soil Sci. Plant Nutr. **47**: 75-78 (In Japanese).

Tsushida, T. and K. Shinohara. 1997. Evaluation of physiological functionalities of horticultural products using mammalian cell culture. Nippon Shokuhin Kagaku Kogaku Kaishi **44**: 607-614 (In Japanese).

Wade, H. E., T. Ball, M. Volokita, C. T. Stephens and K. C. Sink. 1989. Plant regeneration from callus-derived protoplasts of Asparagus. J. Amer. Soc. Hort. Sci. **114**: 1019-1024.