Note

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Effects of Osmoticum and Organic Acids in Media on Protoplast Division in Spinach (*Spinacia oleracea* L.)

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Spinach (*Spinacia oleracea* L.) belongs to Chenopodiaceae is a cool-season annual plant of Asiatic origin. It is an important vegetable which contains ascorbic acids, riboflavin, iron and calcium. With the development of plant biotechnology, protoplast culture has been widely applied to cell biology and molecular biology because of its importance in gene transformation, somatic hybridization, and variation of somatic clones. This method is expected to enable the transfer of desirable traits such as stress resistance and low concentration of oxalic acid to the spinach cultivar. In spinach, the successful regeneration of plants from protoplast was first reported by Goto *et al.* [1] who later reported an improved procedure [2]. On the other hand, Nakagawa *et al.* [3] and Fujita *et al.* [4] succeeded in callus formation from protoplast of spinach, but failed to regenerate plants. Recently, Komai *et al.* [5] reported successful plant regeneration from protoplast. Although the protoplast division is affected by several factors such as source materials, composition of the culture media [6], they studied only the effect of the growth regulators in the medium. Previously, we reported the effect of osmoticum and KM8p organic acids on the division of protoplasts but investigated only two kinds of osmoticum (mannitol and glucose) and did not estimate the effect of osmoticum on plant regeneration [2]. In this paper, we report on our study of the effect of the combination of osmoticum and organic acids in the protoplast media on protoplast division.

Mesophyll protoplasts of spinach(cv. Jiromaru) were isolated from young shoots cultured for about 10 days and purified by the method described previously [1]. The basal medium contained half strength modified MS [7] inorganic salts except NH_4NO_3 (200 mg/*l*), KM8p [8] vitamins, 1.0 mg/*l* BA, and 1.0 mg/*l* 2, 4-D. The protoplasts were cultured in the basal medium supplemented with or without KM8p organic acids (20 mg/*l* sodium pyruvate, 40 mg/*l* citric acid, 40 mg/*l* malic acids, and 40 mg/*l* fumaric acid) containing 0.5 M mannitol and 2.0% sucrose, or 0.5 M sorbitol and 2.0% sucrose, or 0.5 M glucose, or 0.5 M fructose. The media containing KM8p organic acids were significantly less effective or inhibitory. The pHs increased after 3 days incubation at 25°C (Data not shown). The protoplasts were cultured in the media for 2 days. In our previous study [2], sucrose had little effect on protoplast division, so we didn't use it as osmoticum in this experiment. The protoplasts were cultured in plastic petri dishes (35× 10 mm) containing 1.0 m*l* medium at 25°C in the dark for a week, and then were placed under fluorescent light (2000 lux) with 16-hr photoperiod.

To examine the relation between glucose and KM8p organic acids, protoplasts were cultured in the basal medium containing 0.5 M glucose, or 0.4 M glucose and 0.1 M mannitol, or 0.3 M glucose and 0.2 M mannitol, or 0.2 M glucose and 0.3 M mannitol, or 0.1 M glucose and 0.4 M mannitol, with or without KM8p organic acids. Plating efficiency(PE), defined as the number of dividing cells per originally plated protoplast, was recorded at 10 days after plating. After about one month of culture, the micro-calli formed in the media containing 0.5 M mannitol, or 0.5 M glucose with or without KM8p organic acids were transferred to the callus formation medium containing

Table 1. Effects of osmoticum and KM8p organic acid (KM8pOA) on the plating efficiency of spinach protoplasts after 10 days of culture.

Composition of medium		Plating efficiency(%)*
0.5 M mannitol		1.56 c ±0.30
0.5 M mannitol	+KM8pOA	0.22 f ±0.20
0.5 M sorbitol		3.62 d ±1.20
0.5 M sorbitol	+KM8pOA	1.38 ef ±0.22
0.5 M glucose		7.61 b ±2.37
0.5 M glucose	+KM8pOA	10.07 a ±1.78
0.5 M fructose		4.94 c ±1.00
0.5 M fructose	+KM8pOA	4.13 cd ±0.62

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

* The percentage of the number of dividing protoplasts against those of originally plated protoplasts after 10 days of culture.

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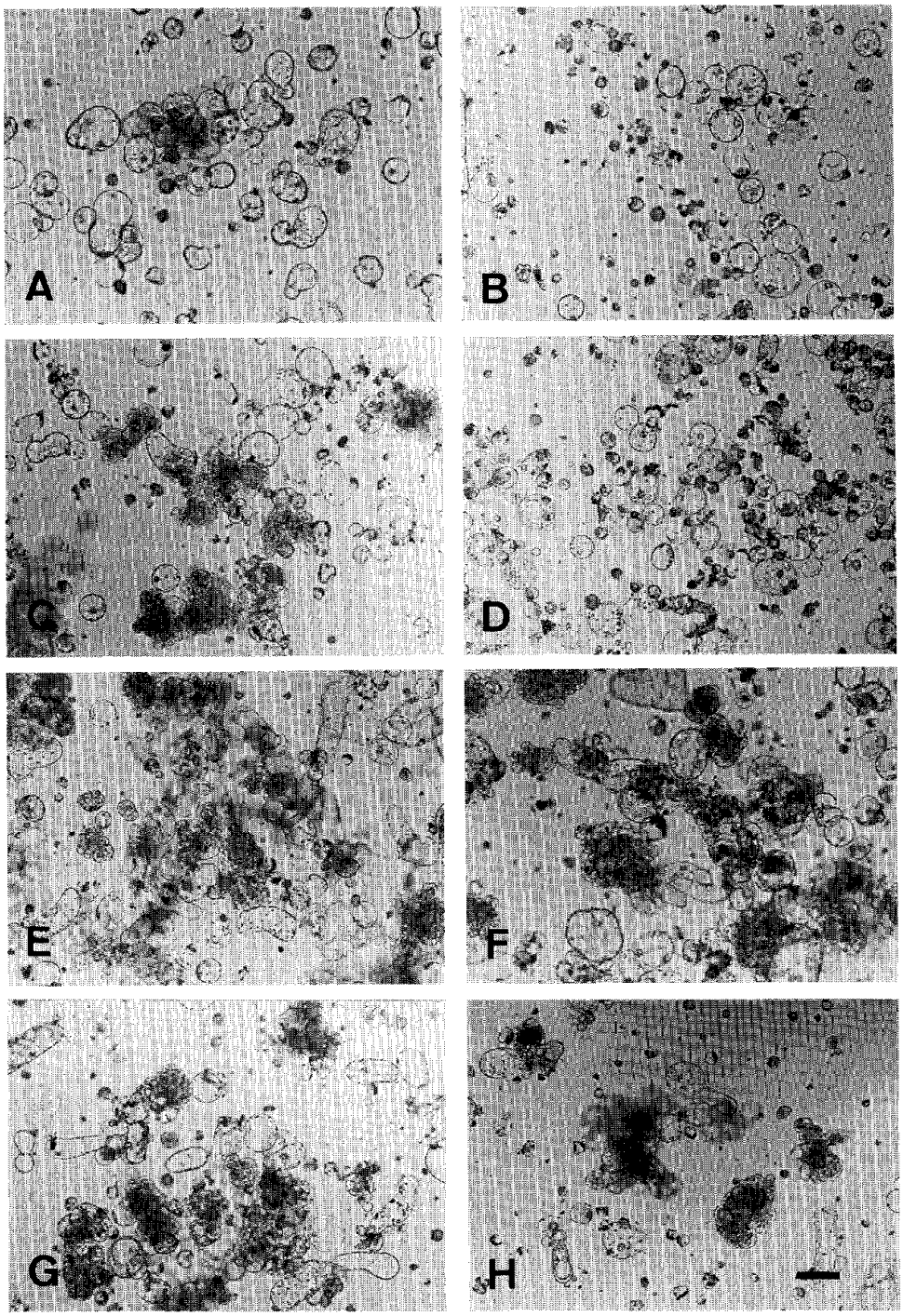

Fig. 1 Influence of various combination of osmoticum and KM8p organic acids on division of protoplasts after 10 days of initial culture.
The protoplasts cultured in the medium containing mannitol (A), mannitol supplemented with KM8p organic acids (B), sorbitol (C), sorbitol supplemented with KM8p organic acids (D), glucose (E), glucose supplemented with KM8p organic acids (F), fructose (G), fructose supplemented with KM8p organic acids (H). Bar=100μm.

1/2 MS inorganic salts, KM8p vitamins, 0.1 mg/l BA, 1.0 mg/l IBA, 1.0 mg/l GA_3, 0.2% sucrose and 0.25% gellan gum. The calli with a diameter of about 2 mm were transferred to the hormone-free MS medium. The number of calli that formed shoots was determined after one month of culture.

The maximum percentage of PE was obtained in the medium containing glucose followed by the media containing fructose, sorbitol, and mannitol (**Table 1**). However, when KM8p organic acids were supplemented in the medium, the protoplast viability and division were considerably affected by the combinations of osmoticum and those organic acids. KM8p organic acids enhanced the protoplast division in the media containing glucose. The combination of KM8p organic acids and sugar alcohol (mannitol and sor-

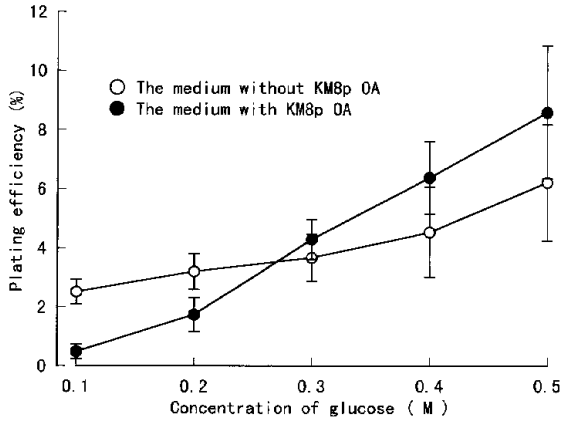

Fig. 2 Effects of the concentration of glucose and KM8p organic acids on plating efficiency (PE) of spinach protoplasts. The osmotic pressures in the media were adjusted 0.5 M.
The PE was defined as the percentage of the number of dividing protoplasts against those of originally plated protoplasts after 10 days of culture. Vertical bars represent SD of means.

bitol) severely inhibited the protoplast division, and only a few protoplasts division were observed even though the protoplasts elongated.

The growth of dividing cells was more efficient when the protoplasts were cultured in the medium containing glucose or fructose compared with sugar alcohol (**Fig. 1**). When mannitol was added to the medium, occasionally some small cells were formed which underwent 1-2 divisions only. The addition of KM8p organic acids into the medium containing glucose stimulated protoplast viability and increased the rate of division.

The effects of concentration of glucose and KM8p organic acids on the PE were showed in **Fig. 2**. The PE increased with higher concentrations in both media with or without KM8p organic acids. The PE in the medium with KM8p organic acids was lower than that without KM8p organic acids until 0.2 M of the glucose concentration, but with a higher concentration of glucose, the PE of the former became higher than that of the latter.

In conclusion, glucose alone was the most conducive to the protoplast division and the growth of colony was more vigorous than with other sugars and sugar alcohols. Similar results were found in *Vitis* [9], *Tanacetum* [10], and *Popula* [11]. However, Nishio *et al.* [12] examined this method for the isolation and culture of protoplasts using various vegetables, and reported that mannitol gave a better result in protoplast culture for most of them, whereas the optimal osmoticum was glucose in tomato, and sucrose in lettuce. Others also have reported the most optimum osmoticum to be sucrose in *Saintpaulia* [13], myoinositol in *Panax ginseng* [14], and sorbitol in *Chrysanthemum* [15]. These results suggested that the protoplast division and growth were often significantly affected by the osmoticum, therefore, it is necessary to find the optimal osmoticum for individual plants. In the present study, the effects of KM8p organic acids on the protoplasts of spinach were considerably different by osmoticum. KM8p organic acids enhanced the protoplast division only when glucose was contained as the osmoticum, whereas they significantly inhibited protoplast division when mannitol or sorbitol was used as osmoticum. In addition, KM8p organic acids induced positive effects on the protoplast division if more than 0.3 M glucose was contained in the medium, though they induced negative effects if less than 0.2 M glucose was contained in the medium. In our previous report, KM8p organic acids did not enhance the protoplast division, whereas they proceeded the division. This difference between present study and previous one might be ascribed to the instability of the media containing KM8p organic acids. Therefore, these results suggest that a combination of the osmoticum and KM8p organic acids would be needed to estimate the effect of individual additives before adding them to the medium. It is unclear how KM8p organic acids influence protoplast culture, but their effect likely relates to the ability of protoplasts to divide.

The micro-calli grown in the medium containing mannitol (minimum protoplast division occurred) or glucose with or without KM8p organic acids (maximum protoplast division occurred) were transferred

Table 2. Effects of osmoticum and KM8p organic acids in the protoplast culture media on shoot regeneration*.

Osmoticum contained in protoplast culture media	No. of calli transplanted A	No. of calli regenerated B	Regeneration ratio (%) B/A×100
0.5 M mannitol	59	5	8.5
0.5 M glucose	71	6	8.5
0.5 M glucose+KM 8 pOA	53	4	7.6

* The micro-calli were cultured on the 1/2 MS medium contained KM8p vitamins, 0.1mg/*l* BA, 1.0mg/*l* IBA, 1.0mg/*l* GA3, 2.0% sucrose, and 0.25% gellan gum. About one month of culture, calli were transplanted on hormone-free MS medium.

on the regeneration medium. The shoot regeneration ratio were unaffected by the osmoticum in the protoplast media (**Table 2**). Also, no difference in the regeneration ratio was observed between the protoplast culture media with and without KM8p organic acids.

Tabei *et al.* [16] reported that the shoot regeneration from the protoplasts of melon was stimulated when glucose was contained in the protoplast medium as osmoticum. In our present study, a combination of glucose and KM8p organic acids enhanced the division of protoplasts significantly, but no obvious effect was observed on the shoot regeneration. However, if the protoplasts were cultured in the medium containing glucose and KM8p organic acids, large numbers of micro-calli would be induced from them and as a result many more regeneration plants would be obtained.

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References

[1] Goto,T., Miyazaki, M., 1992. Plant Tissue Culture Lett., **9**: 15-21.

[2] Goto,T., Miyazaki, M., Oku, M., 1996. J. Japan. Soc. Hort. Sci., **65**: 349-354.

[3] Nakagawa, H., Tanaka, H., Oba, T., Ogawa, N., Iizuka, M., 1985. Plant Cell Reports., **4**: 148-150

[4] Fujita,S., Sugiyama, S., Endo, M., Inada, I., Yamada, O., 1994. Japan. J., Soc. Hort. Sci., **63** (Suppl. 1) : 228-229

[5] Komai,F., Masuda, K.,Harada, T., Okuse, I., 1996. Plans Science, **120**: 89-94.

[6] Potrykus,I., Shillito, R. D. 1988. In "Methods for Plant Molecular Biology." (eds. by Weissbach,A., Weissbach, H.), p. 355-383, Academic Press. INC., San Diego, California.

[7] Murashige,T., Skoog, F., 1962. Physiol. Plant., **15** : 473-497.

[8] Kao,K. N., Michayluc, M. R., 1975. Planta (Berl.) **126**: 105-110.

[9] Mii,M.,Zou,. Y.-M., Sugiyama, T., Yanagihara, S., Iizuka, M., 1991. Scientia Horticulturae, **46**: 253-260.

[10] Keskitalo,M., Kanerva, T., Pehu, E., 1995. Plant Cell Reports, **14**: 261-266.

[11] Wang,Y., Huang, M. R., Wei, Z. M., Suun, Y. R., Chen, D. M., Xu, Z. H., Zhang, L. M., Xu, N., 1995. Plant Cell Reports, **14**: 442-445.

[12] Nishio,T., Sakata, Y., Yamagishi, H., Tabei, Y., Sato, T., Takayanagi, K., 1989. Bull. Nati. Res. Inst. Veg. Ornam. Plants & Tea, Japan, **A3**: 67-96.

[13] Hoshino,Y., Nakao, M., Mii, M., 1995. Plant Cell Reports, **14**: 341-344.

[14] Arya,S., Liu, J. R., Ericsson, T., 1991. Plant Cell Reports, **10**: 277-281.

[15] Endo,M., Fujii, N., Fujita, S., Inada, I., 1997. Plant Biotechnology, **14**: 81-83.

[16] Tabei,Y., Nishio, T., Kanno, T., 1992. J. Japan. Soc. Hort. Sci., **61**: 317-322.