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Plant Regeneration via Shoot Organogenesis from Cotyledons in Two Wild *Cucumis* Species, *C. Figarei* and *C. metuliferus*

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Abstract

Efficient plant regeneration system of *Cucumis figarei* and *C. metuliferus* from cotyledon was developed. Excised cotyledon explants from young seedlings after 3 days of germination were cultured on MS medium containing 30 g/L sucrose, 2.5 g/L gelrite and several combinations of phytohormones. In *C. figarei*, the effect of the combination of BA and IAA on shoot organogenesis was investigated. Adventitious shoots were induced from cotyledonary explants and the highest regeneration frequency was 40% on MS basal medium supplemented with 1 mg/L BA. It was demonstrated that the use of IAA decreased the regeneration frequency, and the application of 2 mg/L IAA suppressed shoot organogenesis completely. Subsequently, other combinations of phytohormones were investigated to improve the regeneration frequency. Combination of 1 mg/L BA and ABA (1 or 2 mg/L) increased the regeneration frequency (ca. 60%), while the combination of 1 mg/L BA and Zeatin (0.5, 1 and 2 mg/L) did not enhance shoot organogenesis and the addition of TDZ (0.2, 0.5, 1 and 2 mg/L) inhibited shoot organogenesis. In *C. metuliferus*, the highest regeneration frequency was 92.5% when cotyledonary explants were cultured on MS medium with 1 mg/L BA and 0.2 mg/L IAA. Lower concentration of IAA (0.2 mg/L) stimulated shoot organogenesis, while a higher concentration of IAA (2, 4 and 8 mg/L) exhibited an adverse effect, and especially, shoot organogenesis was suppressed completely by the addition of 8 mg/L IAA to induce adventitious roots in the greenhouse.

Discipline: Biotechnology
Additional key words: tissue culture

Introduction

Melon (*Cucumis melo* L.) is cultivated over a wide area in the world, and up to now, breeding of melon had been progressing to improve many characters, especially disease and insect resistance. It was reported that several wild *Cucumis* species display a resistance to diseases and insect pests, including the resistance to viruses, *Fusarium* wilt and greenhouse whitefly of *C. metuliferus*[5,7,14], virus resistance of *C. figarei*[13] and root knot nematode resistance of *C. angria*[5,7]. However, in most of the wild species, crossability with cultivated melon is limited[2,9], and it is very difficult to promote the introgression of the resistance to diseases, and/or insect pests of wild *Cucumis* species into cultivated melon by cross-pollination. Under these conditions, cell fusion is considered to be a suitable method for the production of hybrids between cultivated melon and wild

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Cucumis species.

To produce somatic hybrids by cell fusion, it is essential that an efficient protoplast culture system be established and reliable selection methods be also developed. Protoplast culture of melon was already reported by several researcher groups[19,23]. In contrast, attempts at plant regeneration of *C. metuliferus* from protoplasts have been unsuccessful[21], and protoplast culture of other wild *Cucumis* species has not been reported yet. Although the production of somatic hybrids between melon and pumpkin was reported[22], the production of interspecific hybrids between melon and wild *Cucumis* species by cell fusion has not been successful. In the production of somatic hybrids by cell fusion, it is important to develop an efficient regeneration system from protoplasts and reliable methods for the selection of somatic hybrids. Several selection methods of somatic hybrids in cell fusion had already been reviewed by Bajaj[1]. In the review, reliable selection of somatic hybrids by marker genes, e.g. antibiotic resistance genes, introduced into plant through recombinant DNA technology was described. To apply this selection method to the production of somatic hybrids between melon and wild *Cucumis* species, it is essential that selective marker gene(s) be introduced into wild *Cucumis* species. However, the plant regeneration procedure applied to *C. metuliferus*[15,16] and *C. angria*[11] was found to be ineffective practically while that of *C. figarei* has not been reported yet.

In the present study, the use of phytohormones was evaluated to develop efficient plant regeneration in *C. figarei* and *C. metuliferus*. We succeeded in recovering whole plants from cotyledonary explants in *C. figarei* and improving the regeneration system in *C. metuliferus*.

Materials and methods

1) Plant materials and culture conditions

Peeled seeds of *C. figarei* and *C. metuliferus* were surface-sterilized by dipping in a hypochlorite solution containing ca. 1% active chlorite for 10 min, followed by 3 rinses in sterile distilled water. To ensure uniform germination, seeds were placed in a shallow dish and on a wet filter paper with sterile distilled water for 3 days at 28°C in darkness. Afterwards, each cotyledon dissected from seed was cut into 2 pieces and cultured onto MS medium[10] containing 30 g/L sucrose, 2.5 g/L gelrite and phytohormones. The pH of all the media was adjusted to 5.8 prior to autoclaving sterilization at 121°C for 15 min. All the cultures except for the seed germination period were carried out under cool-white fluorescent lamps (ca. 2000 lux) and 16 h photoperiod and at 25°C.

2) Plant regeneration from cotyledonary explants in C. figarei

Media: Combinations of benzyladenine (BA) (0.5, 1 and 2 mg/L) and indole-3-acetic acid (IAA) (0, 0.2 and 2 mg/L) were used to evaluate the effect on shoot organogenesis. Subsequently, effects of 1 mg/L BA combined to several concentrations of abscisic acid (ABA) (0.2, 1, 2 and 4 mg/L), Zeatin (0.5, 2 and 4 mg/L) and thiziazuron (TDZ) (0.2, 0.5, 1 and 2 mg/L) on shoot organogenesis were evaluated. The explants were cultured onto the media in plastic petri dishes ($\phi 90 \times 20$ mm). At 4 weeks after the initial culture, the regeneration frequency was calculated based on the number of explants forming adventitious shoots to that of total explants cultured. The experiment was replicated 3 times, and 10 explants were used in each treatment. Adventitious shoots were transferred to MS medium containing 30 g/L sucrose, 2.5 g/L gelrite and 1 mg/L IAA to elongate shoots and induce adventitious roots. Plantlets with adventitious roots were acclimatized and grew to normal plants.

3) Plant regeneration from cotyledonary explants in C. metuliferus

Media: Combinations of BA (0.1, 0.5, 1 and 2 mg/L) and IAA (0, 0.2, 2, 4 and 8 mg/L) were used to evaluate the effect on shoot organogenesis. At 4 weeks after the initial culture, the regeneration frequency was calculated based on the number of explants forming adventitious shoots to that of total explants cultured. The experiment was replicated 3 times, and 24 explants were used in each treatment. Other conditions were the same as those described above.

Results

1) Production of adventitious shoots in C. figarei

Adventitious shoots of *C. figarei* were induced on media containing only BA as plant growth regulator (Table 1 and Fig. 1-A). Especially, the highest regeneration frequency (43.3%) was achieved at 1 mg/L BA. Lower concentration of BA (0.5 mg/L) was insufficient to promote adventitious shoots, and higher concentration of BA (2 mg/L)

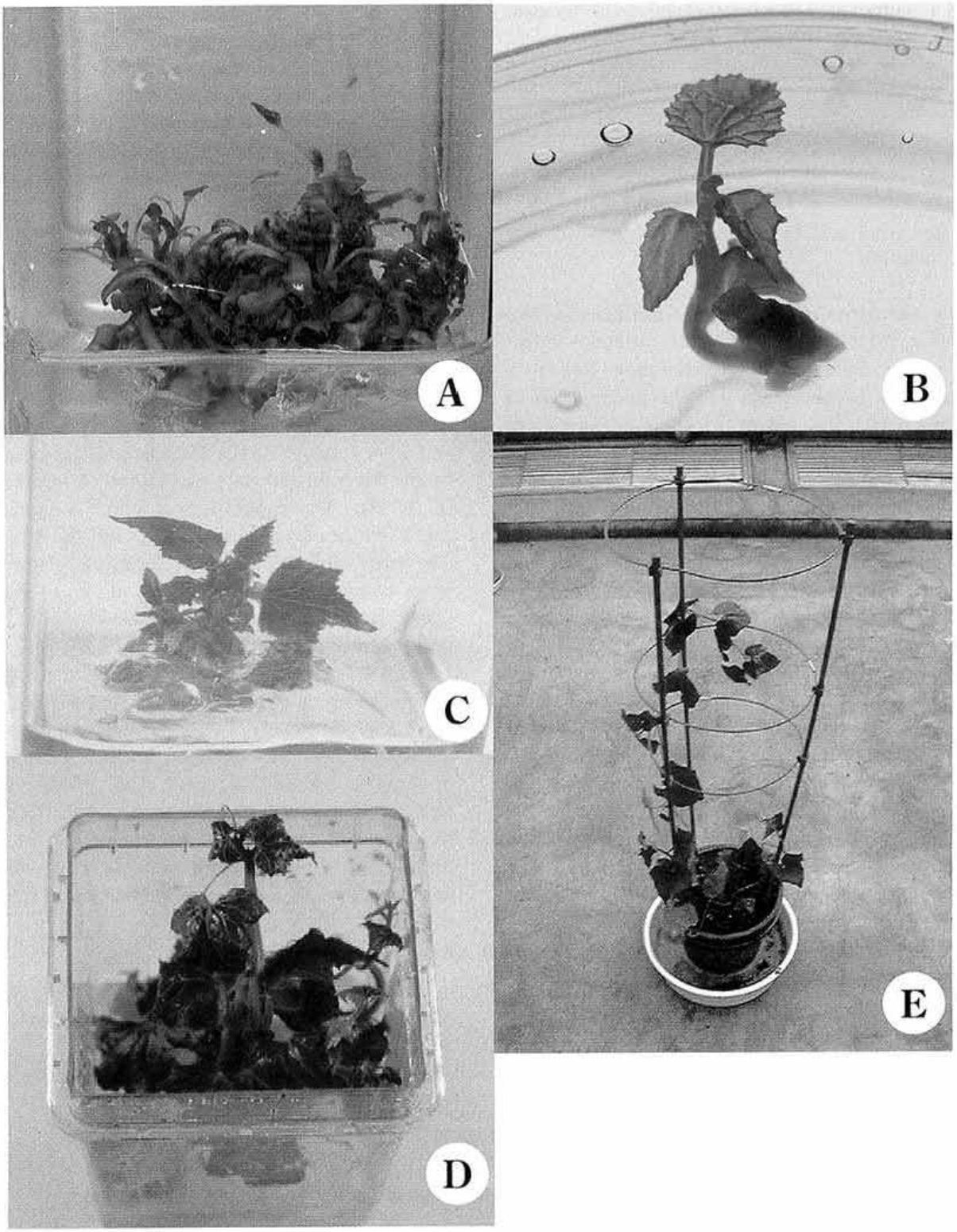

Fig. 1. Shoot organogenesis and plant regeneration in *C. figarei* and *C. metuliferus*
A: Shoot organogenesis from cotyledonary explants in *C. figarei* on MS medium containing 1 mg/L BA and 1 mg/L ABA.
B: Shoot organogenesis from cotyledonary explants in *C. metuliferus* on MS medium containing 1 mg/L BA and 0.2 mg/L IAA.
C, D: Plantlet in *C. figarei* and *C. metuliferus* growing on MS medium containing 1 mg/L IAA, respectively.
E: Regenerated plant in *C. metuliferus* growing in greenhouse.

Table 1. Effect of application of phythormones on shoot organogenesis in *C. figarei*

		IAA (mg/L)		
		0	0.2	2
BA (mg/L)	0.5	10.0 ± 5.8	13.3 ± 3.3	0
	1.0	43.3 ± 5.8	10.0 ± 5.8	0
	2.0	36.7 ± 5.8	10.0 ± 5.8	0

The experiment used 10 explants in each treatment and was triplicated.

slightly decreased the regeneration frequency (36.7%). However, the addition of IAA into the shoot induction media decreased the regeneration frequency remarkably. It was shown that the combination of BA and IAA inhibited shoot organogenesis from cotyledonary explants in *C. figarei*.

As we considered that the regeneration frequency (43.3%) obtained from these experiments was not satisfactory for practical application, other conditions, including the combination of 1 mg/L BA and other phytohormones (ABA, Zeatin and TDZ), were investigated to enhance the regeneration frequency. Combination of 1 mg/L BA and ABA (0.2 to 2 mg/L) increased the regeneration frequency, and the highest regeneration frequency (63.3%) was achieved when cotyledonary explants were cultured with a combination of 1 mg/L BA and 0.2 mg/L ABA (Table 2). However, the addition of 4 mg/L ABA did not improve the frequency of shoot organogenesis. The regeneration frequency under the combination of 1 mg/L BA and 2 mg/L Zeatin was the same as that with 1 mg/L BA only, while the combination of 0.5 mg/L Zeatin decreased the regeneration frequency compared to 1 mg/L BA only (Table 2). TDZ exerted adverse effects on shoot organogenesis in *C. figarei*, and the regeneration frequency decreased remarkably (data not shown). These experiments, indicated that the combination of 1 mg/L BA and 0.2 mg/L ABA promoted shoot organogenesis in *C. figarei* efficiently.

Most of the regenerated shoots were elongated and induced adventitious roots on MS medium supplemented with 1 mg/L IAA (Fig. 1-C). Plantlets acclimatized grew vigorously in the greenhouse.

2) Production of adventitious shoots in C. metuliferus

The use of BA only or of a combination of BA and IAA resulted in the induction of adventitious shoots from cotyledonary explants in *C. metuliferus* (Fig. 1-B). The application of 1 and 2 mg/L BA could induce adventitious shoots, and the frequencies were 84.4% and 80.0%, respectively. However, lower concentrations (0.1 and 0.5 mg/L) of BA induced adventitious shoots less efficiently than higher ones. Moreover, the combination of BA (0.1, 0.5, 1 and 2 mg/L) and 0.2 mg/L IAA enhanced shoot organogenesis, and the frequencies ranged from 78.8 to 92.5%. Especially, the highest regeneration frequency (92.5%) was obtained under combinations of 1 mg/L BA and 0.2 mg/L IAA. However, a higher concentration of IAA decreased the frequencies gradually, and the addition of 8 mg/L IAA suppressed shoot organogenesis completely at all the concentrations of BA in this experiment (Table 3).

Table 2. Effect of addition of Zeatin and ABA on shoot organogenesis in *C. figarei*

Control[a)]	Zeatin (mg/L)			ABA (mg/L)			
	0.5	2	4	0.2	1	2	4
40.0 ± 5.8	30.0 ± 5.8	43.3 ± 3.3	45.0 ± 5.0	63.3 ± 0	60.0 ± 5.8	60.0 ± 10.0	45.0 ± 5.0

The experiment used 10 explants in each treatment and was triplicated.
a): Explants were cultured on MS medium containing 1 mg/L BA, 30 g/L sucrose and 2.5 g/L gelrite.

Table 3. Effect of application of phytohormones on shoot organogenesis in *C. metuliferus*

		IAA (mg/L)				
		0	0.2	2	4	8
BA (mg/L)	0.1	47.2 ± 19.6	78.8 ± 5.3	35.4 ± 8.0	49.6 ± 4.4	0
	0.5	50.4 ± 12.3	91.1 ± 5.1	62.5 ± 22.0	25.0 ± 17.6	0
	1.0	84.4 ± 4.4	92.5 ± 10.6	85.4 ± 4.2	50.1 ± 8.8	0
	2.0	80.0 ± 7.1	89.6 ± 14.7	66.7 ± 18.0	6.3 ± 8.8	0

The experiment used 24 explants in each treatment and was triplicated.

Most of the regenerated shoots were elongated and induced adventitious roots under the same conditions as those for *C. figarei* (Fig. 1-D). Plantlets of *C. metuliferus* also grew vigorously in the greenhouse.

Discussion

Combination of phytohormones often determines the course of morphogenesis, e.g. shoot organogenesis or embryogenesis. The use of plant growth regulators is essential for the control of morphogenesis in tissue culture. In this report, first of all, the effects of combinations of BA and IAA on shoot organogenesis in *C. figarei* and *C. metuliferus* were analyzed, because BA and IAA have been most often used for shoot organogenesis in melon[3,8,18] and cucumber[17]. Combination of BA and IAA was also reported to be effective for shoot organogenesis in watermelon[4,20].

Shoot organogenesis and embryogenesis in *C. figarei* had not been reported yet, and *C. figarei* was considered to be a recalcitrant plant for plant regeneration from tissues. In this report, the regeneration system of *C. figarei* was investigated and developed as in the case of *C. metuliferus*. When cotyledons 3 days after forced sprouting were cultured in the presence of BA (0.5, 1, 2 mg/L) only or in combination with 0.2 mg/L IAA and BA (0.5, 1, 2 mg/L), several adventitious shoots were induced. Addition of IAA into the shoot induction medium suppressed shoot organogenesis. In some regeneration systems via shoot organogenesis in melon and cucumber, it was reported that the utilization of auxin was not essential[3,17]. In the tissue culture of *C. figarei*, the combination of cytokinin and auxin had tended to stimulate callus proliferation, and efficient shoot organogenesis was not achieved, because the conditions of shoot induction from dedifferentiated callus and proliferation were not favorable. Subsequently, BA was combined with several kinds of phytohormones to improve the regeneration frequency. It was found that the combination of BA and ABA stimulated shoot organogenesis from cotyledons 3 days after forced sprouting. Recently, it has been reported that the addition of ABA improved the regeneration frequency via shoot organogenesis and somatic embryogenesis[6,17]. However, the mechanisms of action of ABA in tissue culture have not been elucidated. One of the assumptions was as follows: the induction of ABA when plants experienced several stresses, e.g. low and high temperature, desiccation, microbial infection and injury, conferred a certain degree of tolerance against these stresses[6]. Addition of ABA into the regeneration medium may induce tolerance to tissue culture as a kind of stress.

In *C. metuliferus*, adventitious shoots were induced effectively in the presence of BA only or in combinations of BA and IAA, especially, the highest regeneration frequency was obtained under the combination of 1 mg/L BA and 0.2 mg/L IAA. Orczyk et al.[11] attempted to induce plant regeneration from a leaf of *C. figarei* and *C. metuliferus* in the presence of several concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6(γ, γ-dimethylallylamino)-purine (2iP). Raharjo and Punja[16] succeeded in the induction of shoot callus derived from petioles in the presence of 2,4-D and BA. Punja et al.[15] also succeeded in the induction of adventitious shoots from cotyledon, leaves and petiole. The highest regeneration frequency of 30% was obtained from leaf cultured on MS medium with 5 μM IAA and 5 μM BA. However, Punja et al.[15] failed to achieve plant regeneration when these explants were cultured under a combination of α-naphthaleneacetic acid (NAA) and BA, or 2,4-D and BA. Based on this information, including our results, it was considered that the utilization of IAA and BA was essential for efficient shoot organogenesis in *C. metuliferus*. Although concentrations of BA and IAA reported by Punja et al.[15] and those we used were very close, the regeneration frequency was different between the 2 groups. The difference between these reports was attributed to the difference in the explant used in tissue culture, namely Punja et al.[15] used cotyledon from 8 to 10 days old seedlings and leaves and petiole from 3 to 4 weeks old seedlings, while we used cotyledons 3 days after forced sprouting in our experiment. Cotyledons of mature seed or very young seedlings were used for tissue culture in Cucurbitaceae[3,4,12,17,18,20]. Potential for regeneration of several kinds of explants was examined for shoot organogenesis and embryogenesis in melon, and the regeneration ability of cotyledons of mature seeds was found to be higher than that of cotyledons from 7 days old seedlings and leaves and petiole from 3 weeks old seedlings[18]. Based on these reports, it was assumed that the ability of cotyledons of melon[8,18] and watermelon[4,20] for plant regeneration gradually decreased when the duration of the period after germination increased. For the development of an efficient plant regeneration system in *C. metuliferus*, it is essential to use a combination of BA and IAA as phytohormones for shoot organo-

genesis and young cotyledons 3 days after forced sprouting were also used as explants.

The results of our study suggest that efficient regeneration conditions for *C. figarei* and *C. metuliferus* may enable to produce transgenic wild *Cucumis* species, and contribute to the development of a regeneration system of protoplast culture in *C. figarei* and *C. metuliferus*.

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