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STRESS INDUCED SOMATIC EMBRYOGENESIS IN CARROT AND ITS APPLICATION TO SYNTHETIC SEED PRODUCTION*

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SUMMARY

When apical meristems of carrot (*Daucus carota* L. cv. US-Harumakigosun) seedlings were cultured on hormone-free Murashige and Skoog's (MS) medium with 0.7 M sucrose or 0.25 —1 mM cadmium ion, then transferred to hormone-free MS medium with 0.1 M sucrose, somatic embryos were formed on the surface of the explants without visible callus formation. Somatic embryos were also formed on malformed seedlings, when carrot seeds were treated with hypochlorite solution at a high concentration and sown on hormone-free MS medium with 0.09 M sucrose. These somatic embryos were fractionated by passing through stainless steel sieves with different pore sizes, encapsulated in calcium alginate gel, and placed in plastic petri dishes under sterile conditions. These synthetic seeds germinated 1 to 2 weeks after the beginning of the culture. In the case of synthetic seeds containing a single embryo, the frequency of the seeds which developed both a radicle and a green bud was about 30-50% in large embryos induced by the treatment with sucrose, cadmium or sodium hypochlorite, and about 15% in 2,4-D induced embryos. When 2,4-D induced embryos were encapsulated and sown, numerous secondary and tertiary embryos were formed but they did not develop into normal seedlings.

Key words: cadmium ion; *Daucus carota*; induction; osmotic stress; somatic embryos; synthetic seeds.

INTRODUCTION

Somatic embryogenesis can be induced in a number of dicotyledonous and monocotyledonous species by transferring explants from auxin containing medium to auxin-free medium (2,7,9,10,13,14,19,22). So far, only auxin is known to play an essential role in the induction of somatic embryogenesis, and some biochemical and physiological analyses of somatic embryogenesis were carried out using carrot (4,16,17,21). However, these studies were concerned with a part of the developmental process going from the formation of embryogenic induction in a strict sense. This was due to the fact that auxin exerts various physiological effects, making it difficult to detect only changes which are really related to embryo induction.

It has been reported that somatic embryogenesis in *Helianthus annuus* and *Carica papaya* was stimulated by culturing their explants on medium containing 2,4-D and a high level of sucrose (6-12%) (5,12) and that adventitious shoot formation in tobacco callus required osmotic stress (1). Recently, we showed that somatic embryogenesis in carrot could be induced by giving osmotic stress without hormonal treatment (11). Therefore, we examined effects of several other stresses on the induction of somatic embryogenesis in carrot. In this paper, we report some positive effects of different stresses on embryogenesis as well as their application to synthetic seed production.

MATERIALS AND METHODS

Induction of Somatic Embryogenesis

Daucus carota L. cv. US-Harumakigosun was used as a plant material throughout this work. One-week old seedlings grown on vermiculite were sterilized with 10% sodium hypochlorite solution (a final concentration of available chloride was 1%) for 15 min, then rinsed 3 times with sterilized distilled water. Apical meristems (1 mm) were separated from the seedlings and cultured in petri dishes (6 cm in diameter) containing 20 ml each of Murashige and Skoog's Gelrite (0.2%) medium (hereafter referred to as MS medium) (15) to which no plant growth regulator was added. In a treatment with osmotic stress, sucrose concentration was increased to 0.7 M. Explants cultured on this medium for 2 weeks were then transferred to MS medium with 0.1 M sucrose. To treat the explants with cadmium ion, cadmium chloride was added to MS medium with 0.09 M sucrose at concentrations ranging from 0.25 to 1.0 mM. Explants cultured on these media for 1 to 3 weeks were then transferred to cadmium-free MS medium with 0.09 M sucrose.

For the treatment with hypochlorite, carrot seeds were immersed in a sodium hypochlorite solution (available chloride concentration was 6%) for 1 hour, washed 3 times with sterilized distilled water, then sown on hormone-free MS medium with 0.09 M sucrose.

One month after the start of culture, explants with somatic embryos were suspended in liquid MS medium with 0.09 M sucrose and subcultured every 2 to 4 weeks on a gyratory shaker (70 rpm). Somatic embryos thus obtained were used for further experiments for improving the quality of artificial seeds.

Apical meristems obtained from 1-week old seedlings were cultured on MS medium containing 2,4-D (1 mg/l) and 0.09 M sucrose and subcultured at 2-week intervals. Cell clumps of 63-37 μm in diameter were obtained by passing the suspension through stainless steel sieves (37 and 63 μm in pore size) and washed twice with fresh hormone-free MS medium. The cell clumps (0.1 ml of packed cell volume at 100 ×*g*) were resuspended in 100 ml of hormone-free MS medium and cultured for 2 to 4 weeks to obtain somatic embryos.

Production and Cultivation of Synthetic Seeds

Somatic embryos obtained by the 4 different treatments were divided into 3 categories of size (0.25-0.50, 0.5-1.0, 1.0-2.0 mm in diameter) by passing them through stainless

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steel sieves with appropriate pore sizes. Somatic embryos were washed twice with fresh hormone-free MS medium containing 0.09 M sucrose and collected by centrifugation at 100 $\times g$. They were mixed with 3% (w/v) sodium alginate solution and the mixture was added drop by drop into 100 mM $CaCl_2$ solution with a pipette. After 30 to 60 min, the drops were gelled completely and the capsules were washed twice with fresh hormone-free MS medium. After the capsules were immersed 30 min in the MS medium, only those with a single-embryo were collected and put into empty plastic petri dishes (9 cm in diameter) under sterile conditions. Some of the seedlings germinated in the Petri dishes after 3 to 4 weeks incubation and were transplanted in an open plastic tray filled with vermiculite moistened with plain water.

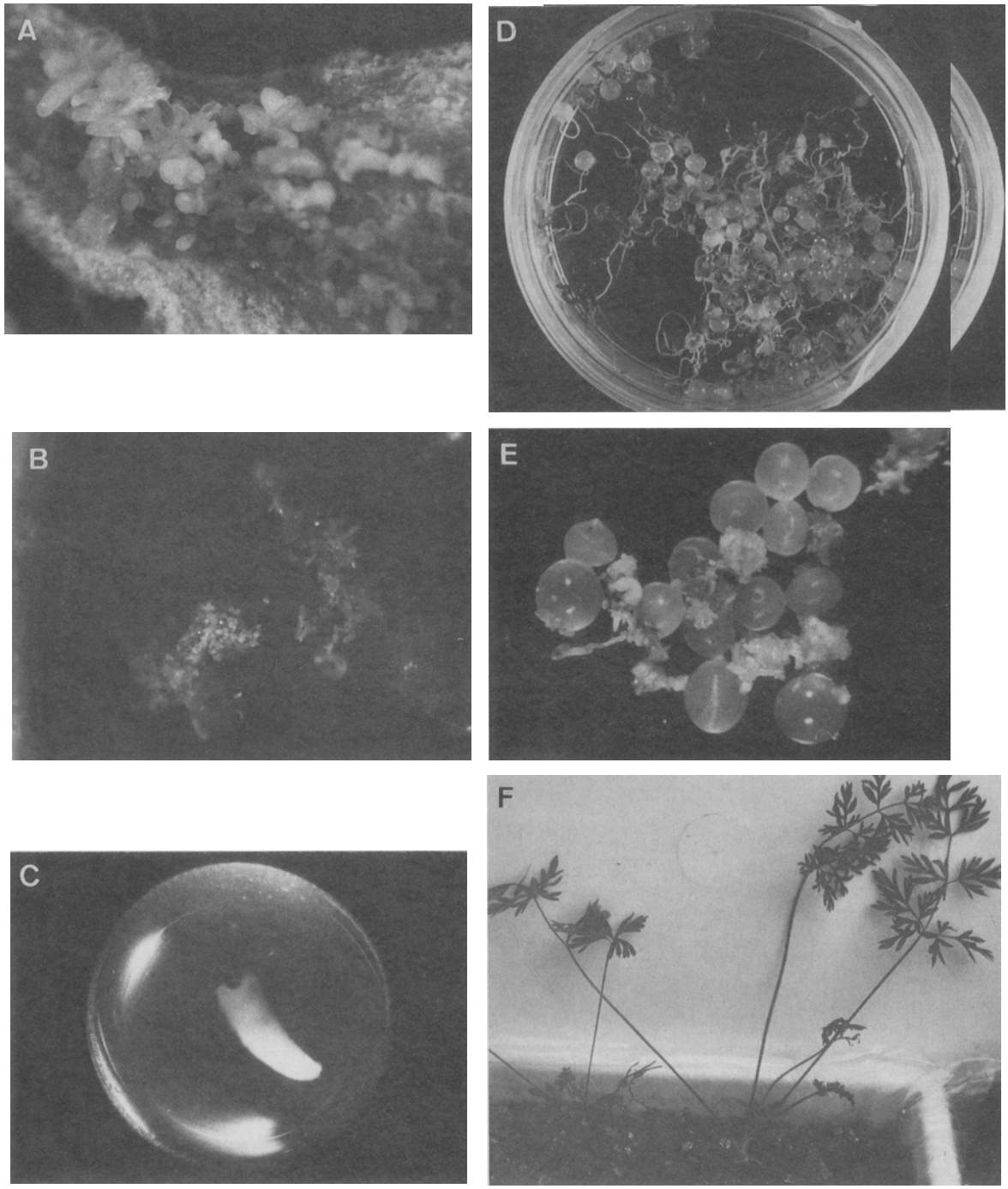

FIG. 1. A) Somatic embryo formation on the surface of a slightly developed leaf of carrot shoot meristem explant treated with sucrose at 0.7 M. (× 25) B) Somatic embryo formation on a malformed seedling after the treatment with hypochlorite solution. (× 4) C) A synthetic seed containing a single carrot somatic embryo (× 10) D) Carrot synthetic seeds having an elongated radicle and/or a green bud. (× 0.8) E) Masses of small embryos developed from carrot synthetic seeds in which 2,4-D induced somatic embryos were encapsulated. (× 2.2) F) Carrot seedlings developed from synthetic seeds in vermiculite under non-sterile conditions. Young seedlings germinated *in vitro* and were transplanted to vermiculite. (× 0.2)

TABLE 1

EFFECT OF CADMIUM ION ON INDUCTION OF SOMATIC EMBRYOGENESIS[a]

Conc. (mM)	Duration of Treatment (weeks)	% of Explants with Embryos	
		Per Total Explants	Per Survived Explants
0.0	1	0.0	0.0
	2	0.0	0.0
	3	0.0	0.0
	Continuous	0.0	0.0
0.25	1	0.0	0.0
	2	0.0	0.0
	3	4.5	5.5
	Continuous	0.0	0.0
0.50	1	18.0	33.0
	2	38.0	63.0
	3	13.0	29.0
	Continuous	0.0	0.0
0.75	1	8.3	50.0
	2	0.0	0.0
	3	0.0	0.0
	Continuous	0.0	0.0
1.0	1	6.4	67.0
	2	0.0	0.0
	3	0.0	0.0
	Continuous	0.0	0.0

[a]Apical meristems were cultured for 1 to 3 weeks or continuously on hormone-free MS Gelrite medium with cadmium chloride at the concentrations indicated, then the explants were transferred to hormone-free MS Gelrite medium without cadmium ion. Each value indicates a percentage of explants producing somatic embryos 6 weeks after the transfer or 6 weeks after the start of culture in continuous treatment. Experiments were conducted with at least 20 replicates for each treatment and all experiments were repeated at least twice.

All cultures were placed under 16 h light (6,000 lux)/8 h dark conditions, at 25° C, except suspension cultures which were carried out in total darkness at 25° C. The experiments were conducted with 20 to 100 replicates for each treatment and repeated at least twice.

Results

When apical meristems cultured on hormone-free medium with 0.7 M sucrose were transferred to hormone-free medium with 0.1 M sucrose, the first leaves were slightly developed and numerous somatic embryos were formed on the surface of the leaves without visible callus formation 4 weeks after the transfer (Fig. 1A). In this case, almost all of the explants survived till the end of culture, and the percentage of explants producing somatic embryos was about 70%. Similar phenomenon was observed on the explants treated with cadmium ion. Up to 40% of the explants produced somatic embryos using 0.5 mM cadmium ion for 2 weeks (Table 1). Most of the explants died during the culture when treated with cadmium ion at a concentration higher than 0.75 mM, and about 60% of the explants survived after the treatment with 0.5 mM cadmium ion. On the other hand, when carrot seeds were treated with a hypochlorite solution containing 6% available chloride and sown on hormone-free medium with 0.09 M sucrose, about 5% of the seeds produced numerous somatic embryos on malformed seedlings without visible callus formation after 4 weeks of culture (Fig. 1B). In this case, the germination rate was about 60%.

Carrot synthetic seeds were round and about 5 mm in diameter (Fig. 1C). The somatic embryos produced by the 4 methods (2,4-D, sucrose, cadmium ion, or hypochlorite) showed different developmental aspects and germination rates depending on the initial treatment. Radicles and green buds emerged 1 week after the sowing *in vitro* and the number of synthetic seeds with a radicle and/or a green bud increased rapidly with time (data not shown here). After 4 weeks of culture, the number of synthetic seeds having both an elongated radicle and a green bud was higher with the somatic embryos produced by sucrose, cadmium or sodium hypochlorite treatment (Table 2 and Fig. 1D) as compared to that produced by the treatment with 2,4-D. When larger somatic embryos (1.0–2.0 mm) were encapsulated to make synthetic seeds, the frequency of those with a green bud and/or a radicle was high (Table 2). This tendency was observed in all cases regardless of the four different somatic embryo induction methods. When somatic embryos induced by 2,4-D were used, several small embryos developed together as a mass in the synthetic seeds (Fig. 1E). Even when these embryos developed to small seedlings with a green bud, numerous secondary and tertiary embryos differentiated from the seedlings but then ceased development. On the other hand, when somatic embryos induced by the treatment with sucrose, cadmium or sodium hypochlorite were used, more than 50% of the embryos developed into seedlings with a green bud, but most of the seedlings had cotyledons with aberrant morphology. Some of the seedlings were transplanted and cultivated in vermiculite under non-sterile conditions with further development (Fig. 1F).

Discussion

In this report, we showed that carrot somatic embryogenesis could be induced by the treatment of apical meristems with cadmium ion at concentrations of 0.25 to 1.0 mM and from seeds treated with concentrated sodium hypochlorite solution. These chemicals are known as stress inducers in plants. In addition to cadmium ion, we recently found that treating

TABLE 2

CONVERSION RATES OF SYNTHETIC SEED[a]

Induction Method of Somatic Embryos	Size of Somatic Embryos (mm)	% of Seeds with		
		Radicle	Green Bud	Radicle and Green Bud
2,4-D	0.25 – 0.5	10	20	8
	0.5 – 1.0	19	35	14
	1.0 – 2.0	14	44	11
Sucrose	0.25 – 0.5	13	12	9
	0.5 – 1.0	34	40	27
	1.0 – 2.0	43	56	35
Sodium Hypochlorite	0.25 – 0.5	37	35	27
	0.5 – 1.0	54	62	47
	1.0 – 2.0	59	68	51
Cadmium Ion	0.25 – 0.5	0	9	5
	0.5 – 1.0	50	49	40
	1.0 – 2.0	66	64	51

[a]Somatic embryos induced by 4 methods were divided into 3 size categories of embryos and encapsulated in alginate gels. The synthetic seeds were put into empty petri dishes and cultured under sterile conditions. Each value indicates a percentage of synthetic seeds producing radicles and/or green buds 4 weeks after the start of culture. Experiments were conducted with at least 100 replicates for each treatment and all experiments were repeated at least twice.

apical meristems with several heavy metal ions, such as cobalt, nickel, and zinc at concentrations of 0.25 to 1.0 mM induced somatic embryogenesis in carrot (manuscript in preparation). It was also reported that the treatment with osmotic stress (0.5 to 0.9 M sucrose or 0.61 M mannitol + 0.09 M sucrose) induced somatic embryogenesis in carrot explants of apical meristems or cotyledons (11). Besides these facts, it is also known that 2,4-D is one of the most effective compounds to induce somatic embryogenesis in many plant species and an inducer of stress proteins (3). Thus, it seems likely that different physiological stresses trigger the induction of somatic embryogenesis. On the other hand, somatic embryogenesis in *Zea mays, Carica papaya, Helianthus annuus* can be induced by the treatment with auxin under high osmotic conditions (5,12), and shoot formation in tobacco callus requires both osmotic stress and hormonal treatment (1). These facts indicate that the induction of somatic embryogenesis by physiological stress must be mediated through a mechanism other than that directly related to hormonal treatment.

Clonal propagation through shoot culture can be used in a number of plants but is often labor consuming because several steps must be cleared before obtaining plants which can grow in soil. This difficulty may be overcome by using synthetic seeds produced by somatic embryogenesis. Several researchers are now working on the production of synthetic seeds and successful encapsulation of somatic embryos with calcium alginate or other gelling reagents has been accomplished (6,8,18). However, frequency of synthetic seeds growing to normal seedlings (conversion rate) was rather low when somatic embryos induced by 2,4-D treatment were used without further improvement (20). One of the reasons for such a low conversion rate was the quality of the embryos. Embryos induced by a high concentration of 2,4-D (50 μM) did not contain seed storage protein of 11S and grew to normal seedlings only at a low frequency. On the other hand, embryos induced by a low concentration of 2,4-D (10 μM) contained seed storage protein of 11S just as that of zygotic embryos and readily grew to normal seedlings (20). In the course of studies on the stress effects on somatic embryo induction in carrot, we found that the somatic embryos which were induced by various stresses described above (except 2,4-D), grew readily to normal seedlings, but the embryos induced by 2,4-D did not. Therefore, we examined the conversion rate of synthetic seeds produced by 4 different methods. The results indicate that the conversion rate was high in the synthetic seeds containing somatic embryos induced by osmotic stress, cadmium or sodium hypochlorite as compared to those induced by 2,4-D. Thus, stress-induced somatic embryos could provide valuable material for synthetic seed production.

As far as carrot is concerned, there is no report demonstrating the presence of storage proteins. We examined the patterns of glyco-proteins binding to Concanavallin A (ConA-binding proteins) by staining with ConA-peroxidase after SDS-polyacrylamide gel electrophoresis of total proteins of somatic embryos. The pattern obtained with somatic embryos was quite similar to that with zygotic embryos, and no difference in the pattern among variously induced somatic embryos was noted (data not shown). It remains to be clarified why the conversion rate of synthetic seeds was low in the case of 2,4-D induced somatic embryos. We are now carrying out a series of detailed analyses of storage like proteins in carrot.

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