Somatic Embryogenesis and Plant Regeneration in Cucumber

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Abstract. The embryogenic capacity of seven cucumber (*Cucumis sativus* L.) cultivars was examined by tissue culture of cotyledon, young first-leaf, and internode explants. Somatic embryogenesis frequencies differed significantly among the tested cultivars, and 'Fushinarimidori' produced the highest number of embryos from either cotyledons or young first leaves. Cotyledon- and first-leaf-derived calluses produced more embryos than calluses from internodes. Somatic embryos were induced from 'Aonaga F₁' internodes. With relatively high sucrose levels (6% and 9%) in the initiation medium, the frequency of embryogenic callus formation from 'Fushinarimidori' cotyledon explants was >90%. The highest yield of somatic embryos occurred in cultures initiated with high sucrose levels (9% or 12%), although 12% sucrose inhibited callus formation and growth. Somatic embryos germinated in a basal liquid medium supplemented with 0.5% activated charcoal, and they developed into well-shaped, healthy plantlets on semisolid medium with 1% sucrose.

Since Malepszy et al. (1982) reported the formation of somatic embryos in cucumber, researchers have made remarkable progress in somatic embryogenesis. Somatic embryos have been induced from various explants, such as cotyledons, hypocotyls, true leaves, or other explants (Bergervoet et al., 1989; Cade et al., 1988, 1990; Chee, 1990; Chee and Tricoli, 1988; Malepsey and Nadolska-Orczyk, 1983; Rajasekaran et al., 1983; Tabei and Kanno, 1989; Trulson and Shahin, 1986; Ziv and Gadasi, 1986). To our knowledge, however, there is no report on successful plantlet regeneration from internode explants via somatic embryogenesis in cucumber. In general, a low frequency of somatic embryogenesis is common in published reports. Moreover, a relatively high frequency of abnormal embryogenesis, such as disordered bipolarity, embryo death, or arrest of growth in developmental stage, recallus, and vitrification of embryos has been reported for cucumber (Bergervoet et al., 1989; Cade et al., 1988, 1990; Jia et al., 1988; Kim and Janick, 1989; Ladyman and Girard, 1992; Ziv and Gadasi, 1986).

In our study, we investigate the influence of cultivar, explant source, and sucrose concentration on embryogenic callus formation and somatic embryogenesis.

Materials and Methods

Seven cucumber cultivars were used in Expt. 1 ('Aonaga F₁', 'Aonagajibai', 'Chikanarishiyou', 'Fushinarimidori', 'Hokushin',

'Shimoshirazu', and 'Yoshinari'); only 'Fushinarimidori' was used in Expt. 2.

Seeds were surface-disinfested for 15 min in 1.0% sodium hypochlorite solution with 3 drops Tween 20/100 ml, followed by three rinses in sterile distilled water. For germination, seeds were placed on basal Murashige and Skoog (1962) medium with 3% (w/v) sucrose, 0.25% (w/v) Gelrite (San-Ei Co., Osaka, Japan), and Gamborg's B5 medium vitamins (Gamborg et al., 1968). All media pH was adjusted to 5.8 with 0.5 N KOH before adding Gelrite and autoclaving for 15 min at 121°C. Unless otherwise stated, cultures were maintained under a 16-h photoperiod at 29 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux (PPF) provided by cool-white fluorescent lamps, at $25 \pm 1^\circ\text{C}$ (80% $\pm$ 5% relative humidity).

Cultivars and explant sources (Expt. 1). Cotyledon, first-leaf, and internode explants from 4-week-old seedlings were excised with a scalpel; cotyledons and first leaves were cut into 10 or 12 disks (4 to 6 mm²), and the internodes were cut into 3- to 4-mm-long sections. All explants were excised on the same day and placed on initiation medium [the basal medium supplemented with 9.0 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 2.7 μM 1-naphthaleneacetic acid (NAA), and 2.2 μM 6-benzylaminopurine (BA)] for 3 weeks. Two replications were used in this experiment, and for each replication, 24 explants were cultured. Calluses were transferred onto embryo induction medium [the basal medium containing 2.7 μM NAA, 2.3 μM kinetin, and 6% (w/v) sucrose] for an additional 2 to 3 weeks of culture. We determined the ability of explants to undergo somatic embryogenesis by counting the number of embryos per explant and the number of calluses forming embryos. Means were compared using Duncan's multiple range test. The interacting effects of cultivars and

explant source on somatic embryogenesis were determined using analysis of variance.

Sucrose concentrations (Expt. 2). Cotyledon explants derived from 6- to 7-day-old seedlings of 'Fushinarimidori' (which produced the most somatic embryos in Expt. 1) were cultured on the initiation medium containing 3%, 6%, 9%, or 12% (w/v) sucrose. After 3 weeks, the proliferating explants with or without calluses were transferred to the embryo induction medium supplemented with 2.7 μM NAA; 2.3 μM kinetin; and containing 3%, 6%, or 9% (w/v) sucrose; or supplemented with 3% sucrose and no plant growth regulators (PGR). The percentage of explants that formed somatic embryos and the number of embryos formed were recorded 5 to 6 weeks after explants were first placed in vitro. Means were compared and evaluated using Duncan's multiple range test or least significant difference. The interacting effects between sucrose levels in the initiation medium and the embryo induction medium on the formation of somatic embryos were determined using analysis of variance.

Development into plantlets. Somatic embryos in the cotyledonary stage with or without visible radicles obtained from the embryo induction medium (after 2 to 3 weeks of culture) were cultured into 200-ml Erlenmeyer flasks containing 15 ml of liquid modified basal medium, supplemented with 0.5% (w/v) activated charcoal (Wako Pure Chemical Industries Co., Osaka, Japan), 1.5% (w/v) sucrose, and 1.5% (w/v) glucose but no PGR. After 1 or 2 weeks of rotatory culture at 1 rpm, the cotyledonary-stage embryos developed cotyledons and roots and grew into whole plants; then they were transferred onto the semisolid basal medium containing 1.0% (w/v) sucrose and held under a 47- $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF with a 16-h photoperiod for 1 week.

Plantlets with well-developed cotyledons and roots were transplanted to $\phi 80 \times 100$ -mm plastic boxes containing autoclaved fine vermiculite wetted with 0.1% (w/v) 6.5N–6P–19K Hyponex (Hyponex Co., Marysville). To harden plantlets, the caps of the containers were opened gradually, until they were completely removed. Hardened cucumber plants were transferred to pots under greenhouse conditions, where they flowered and produced fruit.

Results

Cultivars and explant sources. After 3 weeks of initial culture on the initiation medium, calluses were produced from young first-leaf, cotyledon, and internode explants in all cultivars. The morphology of first-leaf-derived callus was similar to that of cotyledon-derived callus, but it was not similar to that of internode explant callus in size and color. The first-leaf- and cotyledon-derived calluses were yellowish and whitish; the internode-derived callus was larger and greenish. A characteristic translucent and yellowish embryogenic callus tissue with a smooth surface formed and was mixed with the greenish or whitish nonembryogenic callus.

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Significant differences in the capacity of embryogenic callus formation and somatic embryogenesis were observed among the seven cultivars (Table 1). None of the explants from 'Aonagajibai' produced embryogenic calluses. In 'Shimoshirazu', embryogenic calluses and somatic embryos were induced from cotyledon explants but not from first-leaf explants (Table 1). Transferring calluses from either first-leaf or cotyledon segments of 'Fushinarimidori' seedlings onto the embryo induction medium produced the most somatic embryos.

The differing embryo formation capability of the explant sources were evident even when they were excised from the same cultivar (Tables 1 and 2). In 'Fushinarimidori', numerous somatic embryos were produced from the first leaves, whereas 'Aonaga F₁' produced more embryos from cotyledons than from first leaves. Internodes from only one cultivar, 'Aonaga F₁', produced somatic embryos. For cultivars that formed somatic embryos, cotyledons and young first leaves were better than young internodes as explant sources for plant regeneration. The difference in ability to undergo somatic embryogenesis may be due to differences in physiological development or to varying response to the stimulus of the same plant growth regulators. Chee (1990) reported that cucumber cotyledons were better than hypocotyls for somatic embryogenesis.

Sucrose concentrations. Calluses were visible after 3 weeks of 'Fushinarimidori' cotyledon explant culture. Nonembryogenic calluses enlarged with decreasing sucrose concentration in the initiation medium. The highest percentage (100%) of embryogenic callus formed at 6% sucrose (Table 3). Though nonembryogenic calluses were larger and had more vigorous growth when transferred onto the embryo induction medium, embryogenic calluses almost maintained their size and developed farther toward the somatic embryo stage. Somatic embryos were produced on the surface of embryogenic calluses in all treatments.

There was a significant interaction between the sucrose concentration in the initiation medium and the embryo induction medium for the mean percentage of somatic embryo formation and the final number of embryos formed (Table 4). When calluses that formed on initiation medium that contained 12% sucrose were transferred to the PGR-free embryo induction medium and when those that formed on initiation medium with 9% sucrose were transferred to embryo induction medium with 3% sucrose, 6% sucrose, or PGR-free embryo induction medium, many somatic embryos were produced (Table 5). Sucrose concentrations at 6% to 12% in the initiation medium significantly increased the percentage of somatic embryo formation compared to 3% sucrose (Table 6). Also, the number of somatic embryos formed on the embryo induction medium increased quadratically with increasing sucrose concentration in the initiation medium. The percentage of bipolar embryos that formed and were considered normal was correlated quadratically with sucrose level

Table 1. Cucumber cultivar and seedling explant differences in ability of embryogenic callus formation and somatic embryogenesis.^z

Cultivars	First leaves		Cotyledons		Internodes	
	Mean % embryogenic callus formation ^y	Mean total no. embryos formed ^x	Mean % embryogenic callus formation ^y	Mean total no. embryos formed ^x	Mean % embryogenic callus formation ^y	Mean total no. embryos formed ^x
Aonagajibai	0 a	0 a	0 a	0 a	0 a	0 a
Shimoshirazu	0 a	0 a	17 b	13 bc	0 a	0 a
Chikanarishiyou	8 b	5 ab	2 a	3 ab	0 a	0 a
Aonaga F ₁	10 b	10 a-c	15 b	20 cd	17 b	16 b
Hokushin	10 b	16 bc	15 b	16 c	0 a	0 a
Yoshinari	25 c	22 c	19 b	21 cd	0 a	0 a
Fushinarimidori	33 d	72 d	23 b	30 d	0 a	0 a

^zData are means over two replications with four flasks (six explants per flask) per replication. Mean separation within columns by Duncan's multiple range test at $P \leq 0.05$.

^yPercentages (%) of embryogenic callus formation were taken after 3 weeks of culture on the initiation medium. Percentages were transformed into radians {radians = arcsin [square root (proportion)]} for statistical analysis; nontransformed percentages are presented.

^xThe number of embryos was counted after an additional 2 to 3 weeks of culture in the embryo induction medium.

Table 2. Analysis of variance for the effects of cultivars and explant sources on the formation of embryogenic calluses and somatic embryos.

Variable	Source	df	F
Mean embryogenic callus formation ^z	Cultivar	6	47.685**
	Explant source	2	115.103**
	Interaction	12	21.743**
	Error	21	
Mean total no. of embryos formed	Cultivar	6	49.541**
	Explant source	2	58.665**
	Interaction	12	24.241**
	Error	21	

^zPercentages (%) of embryogenic callus formation were transformed into radians {radians = arcsin [square root (proportion)]}.

**Significant at $P \leq 0.01$.

in the initiation medium, with optimal concentrations at 9% to 12%.

Discussion

Our results agree with earlier findings that a relatively high sucrose level in the culture medium has a dramatic effect on somatic embryogenesis (Keller et al., 1975; Tsay et al., 1986; Zhou and Yang, 1981). In addition, relatively high sucrose concentrations in the initiation medium promoted greater frequencies of somatic embryogenesis (Close and Gudeman, 1987; Finer, 1987; Guedira et al., 1989; Lu et al., 1982; May and Trigiano, 1991) or parthenogenesis (Muren, 1989) in other plant species than did lower concentrations.

In our study, the elevated concentrations of sucrose added to the embryo induction medium did not show a significant effect on the percentage of somatic embryo formed or on the final number of embryos formed (Table 6). We found significant differences in the percentage of bipolar embryo formation among the embryo induction media when three sucrose concentrations were tested. Bipolar embryo formation decreased sharply with increasing sucrose concentration in the embryo induction medium. Significant differences also were observed for the PGR-free embryo induction medium (3% sucrose) and PGR-containing embryo induction medium (3%

sucrose) (Table 4). Sucrose at 9% in the embryo induction medium decreased the total number of embryos and the number of normal embryos; it also suppressed the growth and development of somatic embryos in the later stages. Kim and Janick (1989) reached a partly similar conclusion (i.e., higher levels of sucrose in embryo induction medium reduced frequency of embryogenesis and restricted germination of somatic embryos from immature zygotic embryos). Additionally, Chan-

Table 3. Effects of sucrose concentrations in the initiation medium on the formation of embryogenic calluses from cotyledons of 'Fushinarimidori' cucumber.^z

Sucrose concn (% w/v)	Mean no. explants forming embryogenic callus	Mean % embryogenic callus formation ^y
3	19 a	58 a
6	32 b	100 c
9	29 b	91 b
12	16 a	51 a

^zMean separation within columns by Duncan's multiple range test at $P \leq 0.05$. Data are means over three replications with four flasks (eight explants per flask) per replication.

^yFor statistical analysis, percentages (%) of embryogenic callus formation were transformed into radians {radians = arcsin [square root (proportion)]}; nontransformed percentages are presented.

Table 4. Analysis of variance for the effects of sucrose concentrations on cucumber somatic embryo formation in the initiation and embryo induction media.^z

Sucrose concn	df	Mean % embryo formation ^y	Mean total no. embryos formed	Mean % bipolar embryo formation ^y
Level I ^x	3	30.148**	42.699**	86.709**
Level II ^w	2	0.351 ^{NS}	1.738 ^{NS}	58.248**
I × II	6	6.108**	4.246*	5.161**
Error	12			
Level I	3	65.170**	46.897**	135.066**
PGR ^v	1	3.650 ^{NS}	7.184*	78.768**
I × PGR	3	8.356**	7.011*	2.206 ^{NS}
Error	8			

^zEmbryo formation was observed from calluses 2 to 3 weeks after transfer onto the embryo induction medium.

^yPercentages were transformed into radians {radians = arcsin [square root (proportion)]}.

^xSucrose concentrations were 3%, 6%, 9%, and 12% in the initiation medium.

^wSucrose concentrations were 3%, 6%, and 9% in the embryo induction medium.

^vEmbryo induction medium (3% sucrose) supplemented with or without plant growth regulators (PGR).

Table 5. Effects of sucrose concentrations in the initiation and embryo induction media on somatic embryo formation in 'Fushinarimidori' cucumber.^z

Sucrose concn (% w/v)		Mean % embryo formation ^y	Mean total no. embryos formed
Initiation medium	Embryo induction medium		
3	3 ^x	25 a	28 a
	3	46 ab	46 a
	6	25 a	25 a
	9	42 a	45 a
6	3 ^x	79 cd	81 b-d
	3	67 bc	73 b
	6	100 e	103 d-f
	9	88 c-e	92 b-e
9	3 ^x	100 e	124 f
	3	100 e	109 ef
	6	92 de	112 ef
	9	79 cd	77 bc
12	3 ^x	100 e	55 g
	3	79 cd	97 c-e
	6	71 bc	96 b-e
	9	88 c-e	84 b-d

^zData are means over two replications with two flasks (six calluses per flask) per replication. Mean separation within columns by Duncan's multiple range test $P \leq 0.05$.

^yFor statistical analysis, percentages of embryo formation were transformed into radians {radians = arcsin [square root (proportion)]}; nontransformed percentages are presented.

^xContains no plant growth regulators.

Table 6. Effects of sucrose concentrations on somatic embryogenesis from cotyledon-derived calluses of 'Fushinarimidori' cucumber.^z

Sucrose concn (% w/v)	Mean % embryo formation	Mean total no. embryos formed	Mean % bipolar embryo formation
Initiation medium			
3	34	144	23
6	83	349	29
9	93	421	37
12	84	430	46
Significance			
LSD _{0.05}	12.0	59.7	2.6
Linear	NS	NS	**
Quadratic	NS	*	*
Embryo induction medium			
3 ^y	76	387	46
3	73	324	36
6	72	336	28
9	74	298	25
Significance			
LSD _{0.05}	NS	64.8	2.0

^zEmbryos were obtained on embryo induction media 2 to 3 weeks after culturing initiation media-formed calluses.

^yContains no plant growth regulators.

^{NS,*,**} Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

dler and Beard (1983) showed that lower levels of sucrose improved the germination of older zygotic embryos in *Helianthus* in vitro. However, Jia et al. (1988) reported that high sucrose (7% to 9%) added into an embryo induction medium was effective for reducing recalling of cucumber cultures and was favorable for synchronizing the embryoids at globular or late globular stage. Chee and Tricoli (1988) reported that 5% sucrose in a germination medium enhanced embryo development, but a higher sucrose concentration (10%) was not beneficial to embryo development. In our embryo induction medium containing 9% sucrose, however, the cotyledons of germinated embryos were vitrified.

Our experiments demonstrate that somatic embryos could be produced efficiently from cotyledon or young first-leaf explants of 'Fushinarimidori' cucumber on the initial medium containing two kinds of auxins. When explants were cultured on the initiation medium with elevated sucrose then on a low-sucrose-containing embryo induction medium, with or without PGR, the number of embryos and the percentage of normal embryo formed increased. A partitioned analysis of variance shows that sucrose level had a significant influence on somatic embryo formation (Table 4). Our data also indicate sucrose concentrations in the initiation medium and in the embryo induction medium interacted regarding expression of somatic embryogenesis in cucumber.

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