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Somatic Embryos Derived from Cotyledons of Cucumber

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Abstract. **Two studies were conducted to test the effects of various tissue culture media on somatic embryogenesis from cotyledon tissue of cucumber (*Cucumis sativus* L.). The two best media for embryo initiation were Murashige and Skoog (MS) salts and vitamins containing either 1 or 2 mg 2,4-D/liter and 0.5 mg kinetin/liter. In the second study, embryos developed more normally. More plantlets developed when tissue was removed from the initiation medium after 3 weeks and transferred to MS containing 1 mg NAA/liter and 0.5 mg kinetin/liter for 3 weeks, rather than leaving the embryos on a medium containing 2,4-D. Histological evidence indicated that the embryos were multicellular in origin. Charcoal in the maturation medium inhibited embryo development. Chemical names used: (2,4-dichlorophenoxy)-acetic acid (2,4-D); *N*-(2-furanylmethyl)-1*H*-purine-6-amine (kinetin); 1-naphthaleneacetic acid (NAA).**

Regeneration of plants from cucumber somatic tissue can be accomplished either through organogenesis or embryogenesis. Organogenesis was first reported by Maciejewska-Potapczykowa et al. (1972). They successfully regenerated shoots and flowers from stem pieces of cucumber. There have been numerous other reports in the literature on organogenesis in cucumber, but much of the work has been difficult to repeat, and in general the frequency of regeneration is low (Cade et al., 1990; Novak and Dolezelova, 1982; Sekioka and Tanaka, 1981; Wehner and Locy, 1981).

Research on cucumber somatic embryogenesis has shown more promise. Malepszy and Nadolska-Orczyk (1983) were the first to report embryogenesis in cucumber, as well as the first successful plant regeneration from somatic embryos of cucumber (Nadolska-Orczyk and Malepszy, 1984). Orczyk and Malepszy (1985) later refined the techniques and succeeded in regenerating a few plants from protoplast cultures. Others (Jia et al., 1986; Trulson and Shahin, 1986; Ziv and Gadasi, 1986) also reported some success regenerating embryos from cotyledonary callus and/or protoplasts. Chee and Tricoli (1988) were able to obtain mature cucumber plants from leaf explants in liquid culture. The main limitation of cucumber embryogenesis thus far has been the high frequency of abnormal embryos, combined with a low frequency of plant regeneration.

The objective of this research was to expand the current information on somatic embryogenesis in cucumber by studying the effects of growth regulator concentrations in the medium on embryo initiation, and the effect of auxin source and charcoal addition on plantlet development from embryos.

Materials and Methods

Tissue source

Four breeding lines and cultivars (hereafter referred to collectively as cultigens) were used in the experiments. 'Straight

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Table 1. Analysis of variance and mean squares for regeneration traits from cotyledon tissue of two cultigens (Straight 8 and Gy 14A) of cucumber placed on 25 combinations of 2,4-D and kinetin for 6 weeks and subcultured to a medium with or without kinetin for 6 weeks.

Source of variation	df	Embryos/ explant	Rooting score[z]	No. plants/ plate	No. plants/ embryo
		With kinetin			
Replication	3	5.07*	7.64**	0.40	0.000
Cultigen (C)	1	2.11	0.06	0.68	0.001
2,4-D	4	10.35**	26.88**	2.19**	0.030
Kinetin (K)	4	1.50	2.43**	0.77	0.006
C × 2,4-D	4	1.36	1.54	0.69	0.004
C × K	4	2.02	1.15	0.74	0.007
2,4-D × K	16	1.40	1.37	0.42	0.004
C × 2,4-D × K	16	1.58	0.83	0.35	0.001
Error	112	1.79	1.33	0.53	0.019
		Without kinetin			
Replication	3	11.65**	2.99	1.68*	0.025*
C	1	0.20	0.39	0.22	0.004
2,4-D	4	8.95*	21.65**	4.01*	0.005
K	4	6.40+	2.49	6.73**	0.066+
C × 2,4-D	4	1.71	1.18	0.95	0.062*
C × K	4	1.49	0.38	0.80	0.085*
2,4-D × K	16	3.05	0.83	2.50*	0.036*
C × 2,4-D × K	16	1.56	0.85	0.32	0.003
Error	112	2.56	1.24	1.13	0.013

[z]Rated 1–9 (1 = no roots, 5 = moderate rooting, 9 = excessive rooting).
+,*,**Significant at P = 0.10, 0.05, and 0.01, respectively.

Eight' and Gy 14A were used in both experiments; 'Marketmore 76' and 'Sumter' were also included in the charcoal/auxin study.

Seeds from the four cultigens of cucumber were surface sterilized in a 50% Clorox (2.6% NaOCl) solution for 30 min. on a gyratory shaker at 100 rpm, rinsed five times in sterile distilled water and placed in 100 × 15-mm plastic petri dishes each containing 20 ml of 1% Bactoagar (Difco, Detroit, Mich.). Dishes were sealed with Parafilm and placed in the dark at 30C for 5 days to allow the seeds to germinate. Following germination, cotyledons were excised from the seedlings, their margins were removed, and six 2 × 2-mm explants were prepared from each cotyledon.

Culture conditions

Both experiments were conducted in a culture room maintained at 22C with fluorescent and incandescent lamps that continuously provided a photosynthetic flux density of 44 $\mu mol \cdot s^{-1} \cdot m^{-2}$ as measured with a cosine-corrected LI-COR LI-185 quantum/radiometer/photometer (LI-COR, Lincoln, Neb.). For dark conditions, the petri dishes were placed in black sateen cloth bags. Cotyledon tissue was subcultured every 3 weeks to prevent browning of the tissue.

Regeneration procedures

Initiation media study. Five explants were placed in 100 × 15-mm plastic petri dishes each containing 20 ml of MS medium (Murashige and Skoog, 1962) with 0.8% agar and supplemented with 3% sucrose. In addition, each dish contained one of five levels (0.0, 0.25, 0.5, 1.0, or 2.0 $mg \cdot liter^{-1}$) of 2,4-D and kinetin in a factorial design. Two petri dishes each with five explants from the same cotyledon constituted a treatment for the initiation media. The cultures were kept in the dark on the above media for 6 weeks. Each cotyledon piece was then cut into two equal pieces and transferred to either MS medium containing 0.5 mg kinetin/liter or no growth regulators for embryo germination and maturation. Cultures were placed under light for 6 weeks, after which the number of embryos was recorded. Embryos were rated at the early germination stage (Fig. 1B)

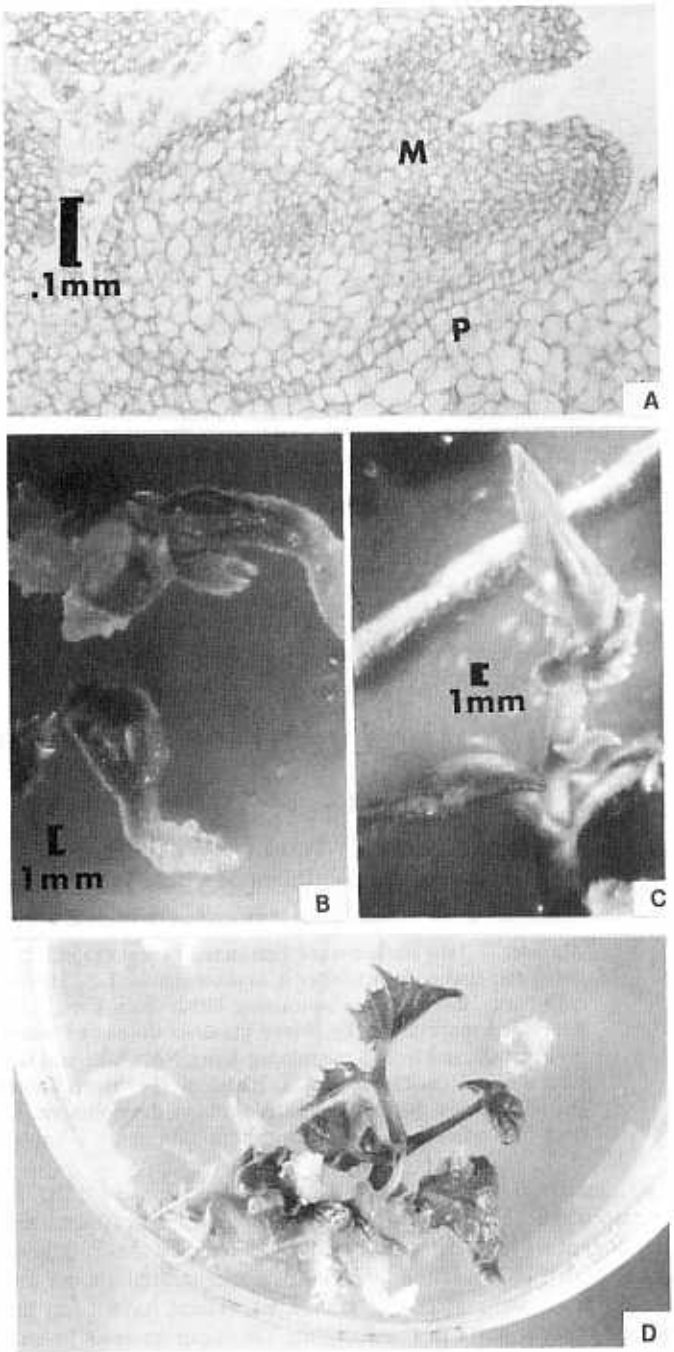

Fig. 1. Stages of embryogenesis in cucumber. (**A**) Heart-stage embryo developing on the surface of cotyledonary divisions after 4 weeks on MS medium with 1 mg 2,4-D/liter and 0.5 mg kinetin/liter (P = protoderm, M = meristematic regions). (**B**) Germinating embryos initiated on MS medium with 2 mg 2,4-D/liter and 0.5 mg kinetin/liter for 6 weeks in darkness and then placed in light on 0.5 mg kinetin/liter. Note the callused roots. (**C**) A normal embryo after 6 weeks in the light on MS medium without growth regulators. (**D**) A plantlet with well-developed leaves and roots after 9 weeks on a medium without growth regulators.

Table 2. Embryo and plantlet production from cotyledon explants of two cultigens of cucumber on 25 combinations of 2,4-D and kinetin for 6 weeks, subcultured to a medium with or without kinetin for 6 weeks[z].

2,4-D concn (mg·liter^{-1})	Kinetin concn (mg·liter^{-1})	With kinetin		Without kinetin	
		Embryos/ explant	Plants/ embryo	Embryos/ explant	Plants/ embryo
Controls[y]		0.0	0.0	0.0	0.0
0.25	0.00	0.1	0.1	0.0	0.0
	0.25	0.2	0.0	0.3	0.0
	0.50	0.2	0.0	0.1	0.5
	1.00	0.2	0.1	0.6	0.0
	2.00	0.4	0.0	0.3	0.0
0.50	0.00	0.1	0.0	0.6	0.1
	0.25	0.6	0.0	1.0	0.4
	0.50	0.5	0.0	0.6	0.0
	1.00	0.4	0.0	0.7	0.0
	2.00	0.5	0.0	0.1	0.0
1.00	0.00	0.0	0.0	0.0	0.0
	0.25	0.7	0.1	1.2	0.1
	0.50	1.0	0.1	2.9	0.3
	1.00	0.6	0.1	0.5	0.0
	2.00	0.6	0.2	0.3	0.0
2.00	0.00	1.1	0.1	0.4	0.0
	0.25	0.4	0.1	0.4	0.2
	0.50	2.7	0.2	3.1	0.2
	1.00	2.6	0.1	1.8	0.1
	2.00	0.8	0.2	0.9	0.2
LSD		1.8	0.2	2.3	0.16
$\bar{x}$		0.5	0.08	0.6	0.08
CV (%)		257	172	251	102

[z]Data are means over four replications, two cultigens, and two plates after 6 weeks on the germination medium.
[y]Controls were of 0 mg 2,4-D/liter, with either 0, 0.25, 0.5, 1.0, or 2.0 mg kinetin/liter.

rather than at earlier proembryonic stages to facilitate data collection. The number of plantlets with well-developed leaves and roots and at least one node was recorded after an additional 3 weeks on MS with or without 0.5 mg kinetin/liter. The experiment was a split-plot treatment arrangement in a randomized complete-block design with four replications. Whole plots were cultigens, and subplots were growth regulators (2,4-D and kinetin).

Charcoal/auxin study. This study was a comparison of two initiation media from the previous study (1 or 2 mg 2,4-D/liter with 0.5 mg kinetin/liter) with a modification of Trulson and Shahin's (1986) CTM 2 medium [MS with (mg·liter^{-1}) 1 NAA, 1 2,4-D, and 0.5 kinetin]. Kinetin was substituted for BA in the CTM 2 medium. All media contained 3% sucrose and 0.8% agar.

After 3 weeks in darkness on one of the initiation media, calli were transferred to either the same medium or to one with 1 mg NAA/liter and 0.5 mg kinetin/liter. The cultures remained in the dark for an additional 3 weeks. Calli from each dish were then cut in half and subcultured to MS medium with 3% sucrose, 1% agar, and 0.5% charcoal, or to the same medium without charcoal for embryo maturation. Embryos remained on MS medium with or without charcoal in the light for 6 weeks before the number of normal and abnormal embryos was recorded. Embryos that lacked a defined bipolar shape or that were fused into clumps were considered abnormal. Plantlet numbers were recorded after 9 to 12 weeks on the maturation media with or without charcoal. Plantlets were then transplanted to vermiculite and covered with plastic bags to prevent desiccation. The experiment was a split-plot treatment arrangement in a randomized complete-block design with two replications of six petri dishes each. Whole plots were combinations of cultigen, initiation medium, and germination medium, and subplots were the maturation medium.

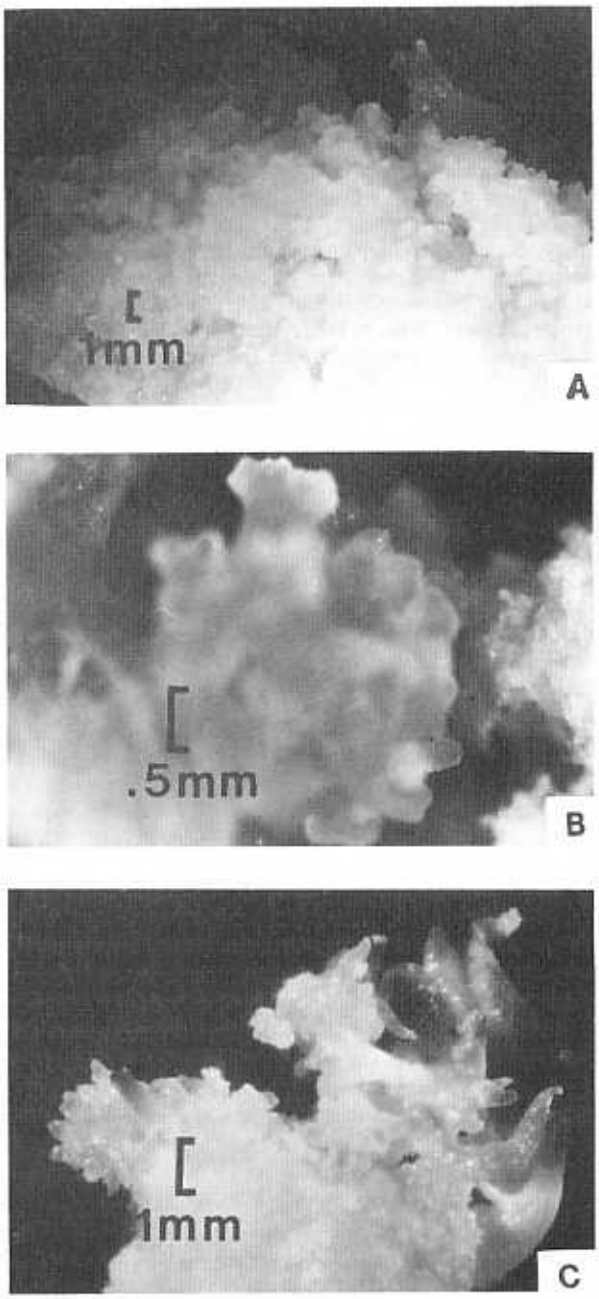

Fig. 2. Early stages of embryonic development. (**A**) Globular tissue proliferation from cotyledon explants after 6 weeks in darkness on MS medium with 2 mg 2,4-D/liter and 0.5 mg kinetin/liter. (**B**) A clump of heart- and torpedo-shaped embryoids after 6 weeks in darkness on MS medium with 1 mg 2,4-D/liter and 0.5 mg kinetin/liter. (**C**) Embryos beginning to elongate and turn green 1 week after removing the explants from the 2,4-D and kinetin medium in darkness and placing them on MS with no growth regulators in light.

Histology

After 6 weeks on the initiation medium, cotyledon and embryo tissues were fixed in formalin-acetic acid-ethanol (FAA).

Table 3. Analysis of variance for embryo and plant production from cotyledon tissue of four cucumber cultigens placed on one of three initiation media for 3 weeks, transferred to one of two germination media for 3 weeks, and subcultured onto MS medium with or without charcoal for 6 weeks[z].

Source	df	Total embryos (No.)	Normal embryos (No.)	Plants (No.)	Normal embryos (%)	Plants/embryo (%)
Replication	1	0.00	17.48**	0.05	1060.61**	52.60
Whole plot						
Cultigen (C)	3	1520.40*	94.75**	11.43**	2504.80**	249.45**
Initiation (1)	2	3.93	1.95	0.54	97.44	206.25*
Germination (2)	1	36.12	0.32	0.99*	1201.66**	346.67**
C × 1	6	12.95	1.45	0.20	118.74	75.90
C × 2	3	16.86	1.83	1.07*	208.10	58.17
1 × 2	2	8.50	1.26	0.32	70.88	56.18
C × 1 × 2	6	46.66	3.61	0.14	150.42	46.84
Error	23	21.62	4.03	0.32	108.72	37.51
Subplot						
Maturation (M)	1	66.86*	1.38	34.16**	932.72**	4942.73**
C × M	3	82.42**	0.82	9.96**	69.73	246.11**
1 × M	2	3.63	0.49	0.57	20.86	181.39*
2 × M	1	0.05	0.54	0.54	0.56	231.69*
C × 1 × M	6	24.22*	1.08	0.22	22.91	85.47*
C × 2 × M	3	13.47	1.00	0.74*	46.43	50.66
1 × 2 × M	2	50.07*	2.83	0.27	8.52	95.49
C × 1 × 2 × M	6	17.94	1.34	0.14	55.56	55.15
Error	24	10.06	1.43	0.31	79.03	39.10

[z]Data are means over two replications and six plates. Initiation media were MS with (mg·liter^{-1}) 0.5 kinetin and either 1 2,4-D, 2 2,4-D, or 1 2,4-D and 1 NAA. Germination media were the same as the initiation or MS, with 1 NAA and 0.5 kinetin.

*,**Significant at $P = 0.10$, 0.05, or 0.01, respectively.

Tissue was dehydrated in a series consisting of tertiary butyl alcohol, ethyl alcohol, and water, then infiltrated and embedded in Paraplast (Monoject Sci. Div., Sherwood Medical, St. Louis), sectioned serially on a rotary microtome at 11-μm thickness, and affixed to slides using Haupt's adhesive (Jensen, 1962). Slides were stained with either toluidine blue for 10 to 15 min, or with a safranin—crystal violet—fast green staining procedure (Gerlach, 1969).

Results and Discussion

Callus initiation media

A friable, yellowish-white tissue began forming around the cut edges of cotyledon explants of both cultigens after 5 to 7 days on media containing 2,4-D. Explants on the control medium (no growth regulators) stayed green for a short time before turning brown. Explants on MS media containing kinetin but not 2,4-D enlarged and remained green for about 6 weeks, but never proliferated.

The amount of 2,4-D in the initiation medium had a significant effect on the number of embryos per explant. However, kinetin in the initiation medium had no effect on embryo formation and plant development unless the embryos were transferred to a germination medium without kinetin (Table 1). Significantly fewer embryos developed into plantlets when kinetin was absent from the initiation medium (Table 2). The interaction between 2,4-D and kinetin had a slightly significant effect ($P = 0.10$) on plantlet production, but did not affect embryo production (Table 1).

Gy 14A produced embryos over a wider range of concentrations but did not perform as well as 'Straight Eight' (data not shown). However, differences between the two cultigens were not significant, so means over cultigens are presented in Table 2. The most embryos were obtained with 1.0 to 2.0 mg 2,4-D/liter and 0.5 to 1.0 mg kinetin/liter in the initiation medium (Table 2). Although the differences among initiation media for plantlet production were not statistically significant, a higher percentage of embryos started on 1 mg 2,4-D/liter and 0.5 mg kinetin/liter developed into plantlets than on the others (Table 2). Lack of significant differences was partly due to use of only four replications. Root formation was also better when embryos were initiated on a medium containing 1 mg 2,4-D/liter. At 2 mg 2,4-D/liter, the embryos produced roots that were often short and had callus development (Fig. 1B).

Small, globular embryos were observed after 3 weeks on initiation medium containing 2,4-D. Friable, yellow embryogenic tissue that was transferred to the same medium after 3 weeks grew rapidly, giving rise to more embryos. However, it appeared that the most friable tissue was not necessarily the best for embryo formation. The embryos seemed to originate from tissue that formed small globular clumps that broke apart easily (Fig. 2A). Heart- and torpedo-shaped embryos were visible by 6 weeks on some of the media containing 2,4-D (Fig. 2B).

Embryos began to turn green and form roots and shoots after they were removed from the initiation medium and grown in light (Fig. 1 B and C and 2C). There were a few embryos that germinated before removal from 2,4-D, but they were abnormal. Paraffin-embedded sections of developing heart-stage embryos showed a definite separation between embryo and maternal tissue, with a visible protoderm surrounding the embryos (Fig. 1A). The embryos were probably multicellular in origin, since they were fused with the parental tissue over a broad area, rather than being attached by a narrow, suspensor-like organ (Williams and Maheswaran, 1986).

Table 4. Means for embryo and plant production from cotyledon tissue of four cucumber cultigens placed on one of three initiation media for 3 weeks, transferred to one of two media for 3 weeks, and subcultured onto MS medium with no growth regulators for 6 weeks[z].

Concn of init. auxin(s) (mg·liter^{-1})	Germ. auxin(s) (mg·liter^{-1})	Without charcoal			With charcoal		
		Total embryos	Normal embryos	Plants	Total embryos	Normal embryos	Plants
			Gy 14A				
1 2,4-D	Same	11.6	4.4	3.3	25.3	5.9	0.0
	NAA	16.1	6.0	4.2	16.0	5.2	0.2
2 2,4-D	Same	15.4	4.1	2.1	20.9	2.5	0.1
	NAA	12.9	3.9	3.6	27.3	5.3	0.2
1 2,4-D	Same	15.9	3.2	1.6	24.6	4.7	0.0
+ 1 NAA	NAA	15.3	4.8	3.7	16.4	3.4	0.3
			Straight 8				
1 2,4-D	Same	4.3	1.0	0.8	2.0	0.0	0.0
	NAA	2.7	0.9	0.4	1.4	0.5	0.0
2 2,4-D	Same	0.3	0.0	0.0	0.0	0.0	0.0
	NAA	1.1	0.5	0.2	4.3	0.9	0.0
1 2,4-D	Same	6.3	1.0	0.7	2.9	0.1	0.0
+1 NAA	NAA	2.6	1.0	0.7	1.6	0.7	0.1
			Sumter				
1 2,4-D	Same	8.6	2.5	1.9	16.5	2.8	0.0
	NAA	5.1	1.8	1.4	5.8	1.9	0.0
2 2,4-D	Same	18.3	5.7	1.7	8.0	1.9	0.0
	NAA	5.5	1.2	0.8	4.2	0.7	0.0
1 2,4-D	Same	3.3	1.1	0.5	3.0	0.6	0.0
+1 NAA	NAA	6.4	2.6	1.4	10.4	2.8	0.0
LSD (5%)		6.3	2.4	1.1	6.3	2.4	1.1
$\bar{x}$		7.2	1.8	0.6	7.2	1.8	0.6
CV (%)		44	67	88	44	67	88

[z]Data are means over two replications with six plates per replication. Initiation media were all MS with (mg·liter^{-1}) 0.5 kinetin and either 1 2,4-D, 2 2,4-D, or 1 2,4-D with 1 NAA. Germination media were either the same as the initiation media or 1 mg NAA/liter with 0.5 mg kinetin/liter.

Charcoal/auxin study

While auxin source in the germination media did not have an effect on total embryo production, it did have a large significant effect on the percentage of normal embryos produced and the number of plantlets obtained per embryo (Table 3). All three initiation media were similar, both in number of embryos produced on them and percentage of plants obtained (Table 4). 'Marketmore 76' did not perform well on any of the media and, therefore, was excluded from Table 4. In general, removing the immature embryos from media containing 2,4-D after 3 weeks and placing them on a medium containing NAA increased the number of normal embryos and the percentage of embryos that developed into plants.

Ziv and Gadasi (1986) reported that the addition of charcoal in double-layer suspension cultures of cucumber decreased the percentage of abnormal embryoids, possibly by adsorbing excess growth regulators or phenolic compounds or by preventing precocious germination. In this study, however, adding charcoal directly to the maturation medium, while increasing total embryo production, significantly decreased both the number of normal embryos and the rate of conversion to plants. Charcoal in the maturation medium inhibited germination, caused vitrification of the embryos, and made them chlorotic. Problems may have been due to charcoal adsorbing excessive amounts of sugars or exogenous growth regulators that were present when the embryos were transferred to maturation medium. Removing the embryos from the charcoal medium sooner perhaps would allow the embryos to develop more normally. Plantlets were transferred on several occasions to vermiculite in transparent plastic containers, but the roots did not develop fast enough for plants to survive.

It appears that the percentage of normal embryos increases when the explants are removed after 3 weeks from media containing 2,4-D, but the percentage of embryos maturing into plants remains low. More research is needed to determine the optimum stage of embryo development for removal of 2,4-D. In these experiments, all of the embryos, regardless of their growth stage, were taken off of the 2,4-D treatment at the same time. Thus, we expected that some of the embryos would be abnormal or undeveloped due to an improper duration on 2,4-D. Future research might solve this problem by either removing individual embryos from 2,4-D at the correct stage, or finding an alternative auxin, such as NAA or IAA, that may promote more normal embryo development.

Literature Cited

Cade, R.M., T.C. Wehner, and F.A. Blazich. 1990. Effect of explant age and growth regulator concentration on adventitious shoot formation from cotyledonary tissue of cucumber. Cucurbit Genet. Coop. Rpt. (In press.)

Chee, P.P. and D.M. Tricoli. 1988. Somatic embryogenesis and plant regeneration from cell suspension cultures of *Cucumis sativus* L. Plant Cell Rpt. 7:274–277.

Gerlach, D. 1969. A rapid safranin–crystal violet–light green staining sequence for paraffin sections of plant materials. Stain Technol. 44:210.

Jensen, W.A. 1962. Botanical histochemistry. W.H. Freeman, San Francisco.
Jia, S., Y. Fu, and Y. Lin. 1986. Embryogenesis and plant regeneration from cotyledon protoplast culture of cucumber (*Cucumis sativus* L.). J. Plant Physiol. 124:393–398.
Maciejewska-Potapczykowa, W., A. Rennert, and E. Milewska. 1972. Callus germination and growth of tissue cultures derived from cucumber plant organs of four different sex types. Acta Soc. Bot. Poland 41:329–339.
Malepszy, S. and A. Nadolsky-Orczyk. 1983. *In vitro* culture of *Cucumis sativus*. I. Regeneration of plantlets from callus formed by leaf explants. Pflanzenphysiologie 111:273–276.
Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
Nadolska-Orczyk, A. and S. Malepszy. 1984. Cucumber plant regeneration from leaf explants. Bul. Acad. Polish Sci. Biol. 32:11–12.
Novak, F.J. and M. Dolezelova. 1982. Hormone control of growth and differentiation in the in vitro cultured tissue of cucumber (*Cucumis sativus* L.). Biologia 37:283–290.
Orczyk, W. and S. Malepszy. 1985. *In vitro* culture of *Cucumis sativus* L. V. Stabilizing effect of glycine on leaf protoplasts. Plant Cell Rpt. 4:269–273.
Sekioka, T.T. and J.S. Tanaka. 1981. Differentiation in callus cultures of cucumber (*Cucumis sativus* L.). HortScience 16:451. (Abstr.)
Trulson, A.J. and E.A. Shahin. 1986. *In vitro* plant regeneration in the genus *Cucumis*. Plant Sci. 47:35–43.
Wehner, T.C. and R.D. Locy. 1981. In vitro adventitious shoot and root formation of cultivars and lines of *Cucumis sativus* L. HortScience 16:759–760.
Williams, E.G. and G. Maheswaran. 1986. Somatic embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57:443–462.
Ziv, M. and G. Gadasi. 1986. Enhanced embryogenesis and plant regeneration from cucumber (*Cucumis sativus* L.) callus by activated charcoal in solid/liquid double-layer cultures. Plant Sci. 47:115–122.