Cucumber Somatic Embryo Development on Various Gelling Agents and Carbohydrate Sources

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Abstract. Somatic embryos were induced on cotyledonary explants of cucumber (*Cucumis sativus*, L. cv. Clinton). The somatic embryo tissue was induced and multiplied on a semisolid medium. The greatest vitrification and expansion of explants were observed when 0.15% Gelrite was the gelling agent. At the induction stage, explants on a medium gelled with 0.7% agar yielded more somatic embryo tissue, uncontaminated by callus, than explants on either Gelrite (0.15%, 0.3%, and 0.6%) or on 1.4% or 0.35% agar. In contrast to the induction stage, there was no effect of gelling agent on the fresh weight of putative somatic embryo tissue produced during the multiplication stage. When sucrose, glucose, or fructose was the sugar source in the multiplication medium, similar weights of tissue were produced. However, when sucrose was compared with fructose in the presence of either agar or Gelrite as gelling agent, the subsequent germination of the somatic embryos was higher when the tissue was multiplied on Gelrite in the presence of 3% sucrose. Maltose (3%) severely inhibited tissue multiplication.

We were investigating the possibility of using somatic embryos of cucumber as artificial seeds and were interested in maximizing the number of regenerable embryos derived by somatic embryogenesis.

Cucumber somatic embryos have been derived from a variety of explants, e.g., cotyledons (Chee, 1990; Trulson and Shahin, 1986; Ziv and Gadasi, 1986), primary leaves (Chee and Tricoli, 1988), immature zygotic embryos (Kim and Janick, 1989), and hypocotyl segments (Chee, 1990). Somatic embryos produced from immature zygotic embryos had malformed cotyledons and did not regenerate into seedlings (Kim and Janick, 1989). The procedures developed by Trulson and Shaheen (1986) and Ziv and Gadasi (1986), which used cotyledonary explants, led to well-formed embryos that germinated successfully but did not have a multiplication phase of the primary embryos in the protocol. Chee and Tricoli (1988) developed a procedure whereby suspension cultures were established from embryogenic callus derived from the primary leaves of young plants. In our regeneration method a conditioning step in liquid suspension in the presence of abscisic acid has been incorporated, in addition to the induction and multiplication stages on semisolid media.

The object of this work was to determine the effect of gelling agent type and concentration on the induction of somatic embry-

ogenic tissue and to compare the effect of gelling agent type and carbon source during the multiplication phase on somatic embryo development of cucumber. Kim and Janick (1989) suggested that the carbohydrate source may be a critical factor in cucurbit morphogenesis.

Explant source and preparation. 'Clinton' cucumber seeds (*Cucumis sativus*) were sterilized for 10 min in 10% Clorox bleach, to which Tween 80 (three drops per 100 ml; Sigma, St. Louis) had been added. The seeds were rinsed three times in sterile water and left on moist germination paper for 24 h in the dark. The seeds were then planted on MS medium (Murashige and Skoog, 1962) solidified with 1% agar in Magenta boxes (Magenta Corp., Chicago) and left 3 days at a 16-h photoperiod, 26C day/18C night. Bxplants made with a cork borer (0.5-cm i.d.) were taken from the cotyledons and placed on the induction medium.

The induction medium constituents were similar to CTM2 (Trulson and Shahin, 1986) except that the concentrations of plant growth regulators were (mg-liter⁻¹): 1.1 2,4-D; 0.2796 NAA; and 0.226 BAP. Agar (0.35%, 0.7% and 1.4%; Baltimore Biological Laboratories, Cockeysville, Md.) was compared with Gelrite (gellan gum; 0.15%, 0.3%, and 0.6%; Kelco Div., San Diego, Calif.) for suitability as the gelling agent for the induction phase. The volume of agar-gelled media was equal to that of the Gelrite-gelled media (35 ml/petri plate). The explants were left in light 24 h ($\approx 36 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 26C in an incubator.

Multiplication of tissue was carried out on a medium similar to the induction medium, except that NAA was omitted. Somatic embryos (embryogenic tissue), along with a very small amount of callus, were sliced off ex-

plants that had been incubated for 14 days on induction medium (0.7% agar) and placed on multiplication medium. There were two separate experiments in which the following modifications were made to the medium; 1) solidified with 0.7% agar or 0.15% Gelrite, which produce media of comparable firmness (Pasqualetto et al., 1988) in the presence of 3% sucrose, or 2) either fructose (3%), glucose (3%), or maltose (3%) was substituted for the sucrose in the presence of agar (0.7%). About 100 mg of embryogenic tissue was placed onto each petri plate. The tissue was kept under continuous, cool-white fluorescent light ($\approx 36 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 26C. The tissue was transferred after every 4 weeks to fresh multiplication media. At the time of transfer the cream-yellow, smooth embryogenic tissue was separated from hard, slightly pubescent callus tissue that then was discarded. In both experiments, the fresh weight of the putative embryogenic tissue was used to quantify multiplication of somatic embryos. The effect of treatment on tissue water retention was not estimated. There were seven petri plates for each treatment and results were supported in at least one other experiment.

Although there was no significant effect of gelling agent on the weight of putative embryogenic tissue produced during multiplication, the effect of gelling agent on the development and germination of somatic embryos was examined in a separate experiment. Somatic embryos were induced on 0.7% agar and multiplied on media gelled with either Gelrite (0.15%) or agar (0.7%) in the presence of either sucrose (3%) or fructose (3%). The initial amount of embryogenic callus was accurately weighed to provide a means of estimating the number of regenerants per unit of starting material. After 10 weeks on multiplication medium, the embryogenic tissue was suspended in liquid "conditioning" medium in total darkness (28C) on a reciprocal shaker (66 cycles/min). This conditioning medium was similar to the multiplication medium except that it contained 0.2 μM abscisic acid and no gelling agent. After 5 days the suspension was sieved sequentially through steel mesh of 860 μm and 520 μm . The filtrate was centrifuged ($\approx 150 \times g$) for 10 min and the supernatant was discarded. The embryos were resuspended in a volume of MS solution twice that of the bed volume and were plated onto

Table 1. Fresh weight of the total tissue induced on nine cotyledonary cucumber explants after 14 days on an induction medium.^a

Gelling agent and concn (%)	Tissue wt (g)	SE
Agar		
0.35	0.388	0.056
0.70	0.436	0.064
1.40	0.084	0.011
Gelrite		
0.15	0.528	0.073
0.30	0.567	0.081
0.60	No data	

^aData are means of seven plates; nine cotyledonary explants per plate. Each explant was 0.196 cm².

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Table 2. Multiplication of putative somatic embryogenic cucumber tissue on a medium containing four carbohydrate sources and gelled with 0.7% agar.^c

Time in culture (weeks)	Wt of embryogenic tissue (g)							
	Sucrose		Maltose		Fructose		Glucose	
	Mean ^a	SE	Mean	SE	Mean	SE	Mean	SE
0	0.103	0.003	0.104	0.001	0.102	0.002	0.102	0.003
8	5.716	0.495	0.075	0.014	5.652	0.385	5.176	0.620

^aData are means of seven petri plates. In each petri plate ≈ 100 mg (time in culture = 0) of embryogenic tissue was placed.

Table 3. The number of regenerants after cucumber tissue multiplication on either an agar- or Gelrite-gelled medium.

Medium		Total germinated (embryos/50 mg ^a)	Regenerants (no.)		
Sugar source (3%)	Gelling agent		Without roots	Not transferred to soil ^b	Transferred to soil
Sucrose	Gelrite (0.15%)	81	22	23	36
	Agar (0.7%)	11	5	3	3
Fructose	Gelrite (0.15%)	3	1	1	1
	Agar (0.7%)	0	---	---	---

^aTotal shoots (one shoot per embryo) produced from an initial weight of 50 mg of putative embryogenic tissue.

^bRegenerants having a root system of two or fewer roots < 3 cm long were not transferred to soil.

MS medium solidified with 1% agar. Embryos started to germinate ≈ 24 days after transfer onto the hormone-free medium. Seedlings that developed a healthy root and at least three leaves were transferred to soil for slow acclimation to greenhouse conditions (16-h photoperiod, 26C day/18C night).

Induction of embryogenic tissue. Induction was found to be best on 0.7% agar. In this case, the putative somatic embryos that formed around the cut edge were smooth and yellowish and very little non-embryogenic tissue (callus) developed. Gelrite at 0.15% caused excessive expansion of the explants and the tissue became vitrified. In addition, no somatic embryos and very little callus were formed. Vitrification is defined here as glassiness and hyperhydration as described by Debergh (1983). Less expansion and less vitrification occurred on 0.3% and 0.6% Gelrite, but the tissue produced more callus and fewer embryos than that on 0.7% agar. Tissue quality from cultures on 0.35% agar resembled that on 0.6% Gelrite, i.e., well-formed glossy embryos were numerous, but they were surrounded by more callus than tissue on 0.7% agar. More material was harvested from the Gelrite plates (Table 1), but we observed that the embryogenic tissue was less discrete and wetter, and more non-embryogenic callus was inadvertently harvested. Explants on 1.4% agar did not expand, vitrify, or produce callus, but the production of embryos was also very limited (Table 1).

Multiplication of embryogenic tissue. During the multiplication phase, in the first experiment, Gelrite (0.15%) gave about the same fresh weight of embryogenic tissue as agar (0.7%). After 12 weeks on multiplication media, the initial 100 mg of tissue yielded 10.9 g (± 2.3 g) tissue on Gelrite and 11.5 g (± 1.9 g) on agar. Chee (1990), in her report on somatic embryogenesis from coty-

ledonary explants, described the type of embryogenic tissue we observed during the multiplication phase, although conditions and hormones differed between the two protocols. In the experiments reported here, tissue on both agar and Gelrite was translucent and smooth, but on Gelrite the gelatinous material, as described by Chee (1990), appeared to be more dense. However, this characteristic was too difficult to quantify. Maltose inhibited cucumber tissue multiplication (Table 2). It has been shown that maltose significantly increased the number of somatic embryos produced from alfalfa callus culture (Strickland et al., 1987) and from petunia anthers (Raquin, 1983), whereas potato anthers cultured on a medium containing maltose produced significantly fewer embryos than those cultured on sucrose (Batty and Dunwell, 1989). The ammoniacal-nitrogen (NH_4) concentration (15 to 20 mM) was determined to be important for optimum alfalfa embryo production (Strickland et al., 1987) and a similar concentration (20 mM) was used in our multiplication medium.

Regeneration of somatic embryos. In the experiment that compared sucrose with fructose in the presence of either Gelrite or agar, the number of regenerants per 50 mg of primary somatic embryo tissue was recorded 4 weeks after the embryos had begun to germinate. When the multiplication medium contained Gelrite and sucrose, the majority of embryos produced both shoots and roots, about half of which were very vigorous and subsequently were transferred to soil (Table 3). When agar was substituted for Gelrite the number of regenerants decreased substantially. When fructose was substituted for sucrose few embryos germinated from either gelling medium. Germinated embryos that were transplanted into soil and transferred to the greenhouse developed into healthy robust

plants that flowered and produced fruit (Ladyman and Girard, 1990).

These results demonstrate that somatic embryogenic tissue was most successfully induced on a medium gelled with agar (0.7%). When sucrose, glucose, or fructose was the sugar source in the multiplication medium, similar weights of tissue were produced. However, under the defined conditions, when fructose was compared with sucrose in the presence of either agar or Gelrite, the most somatic embryos germinated (per unit of initial embryogenic tissue) when the tissue was multiplied on a medium containing sucrose and gelled with Gelrite. Maltose was deleterious to cucumber tissue multiplication.

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