Chapter 3

Plant Tissue Culture Systems as Instructional Tools in the Biological Sciences

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Introduction

There has been a rapid increase within the last twenty years in the number of researchers involved in the aseptic culture of plant cells, tissues, and organs; that is, "plant tissue culture". The reasons are twofold: (1) important technical developments have allowed for greater reproducibility of experimental results, and (2) numerous investigations have demonstrated the utility of these techniques in the study of many biological questions especially in genetics, physiology, and development (Chaleff and Carlson 1974; Day 1977; Murashige 1978; Nickell and Torrey 1969; Smith 1974; Sprague et al. 1980; Zenk 1978). Consequently, this exercise has been designed to introduce students to some of the basic techniques of plant tissue culture. Unlike the typical laboratory exercise which has a definite end in mind, this exercise is "open-ended" and suggests a number of lines of possible investigation. Thus, it is hoped that as a result of this exercise students will be encouraged to continue in an area suitable to their own interests. Students should not feel restricted to continue their study with the species chosen for this exercise (maize and carrot) since the various protocols presented can serve as a foundation for further investigation with other species. However, students should realize that for successful work with any plant species it may be necessary to vary manipulative procedures (method of sterilization, composition of media, and so forth). Fortunately, much of the necessary information has been well-documented in the literature (Street 1977). In the discussions to follow the procedures for initiating and maintaining cultures, and for testing the effects of amino acids on the growth of callus, will be presented separately for maize and carrot. Special terms are defined in Appendix A. The student should be aware that the experiments for both organisms require periodic attention over the course of a few to several weeks, depending upon which aspect is being investigated.

Instructors' Materials

Maize

Culture Initiation

Ears can be removed from either field- or greenhouse-grown plants at 12 through 24 days after self- or cross-pollination. (If facilities are not available for raising your own corn, fresh ears may be purchased from the grocery.) Individual kernels should be immediately removed at random. The immature embryos can then be isolated by first cutting off the base of the kernel and then applying pressure to the crown to force the embryo out. The embryos are then surface-sterilized for five minutes in a 2% solution of sodium hypochlorite

(= 20% solution of Clorox or any comparable household bleach) and rinsed twice in sterile, double-distilled water. The embryos, with their attached dome-shaped scutella intact, should then be aseptically transferred to the culture medium and oriented so that the flat embryonic axis is in contact with the medium, and the rounded scutellar side is exposed. Any embryos damaged during excision should be discarded. Callus can be initiated on the maize medium containing 0.5 to 4.0 mg/l 2,4-D and maintained by subculturing every three to four weeks to media containing the same amount of hormone. All embryonic structures should be removed at the primary subculture so that only callus is transferred. Cultures are incubated at 28–30°C with a 16:8 L:D photoperiod from cool-white fluorescent lamps in combinations providing an intensity of 3000–4000 lux.

Observations on Culture Initiation

After 24 to 48 hours of incubation the dome-shaped scutellum appears to swell. Within 72 hours embryonic growth is evidenced by the elongation of the shoot and/or root. Callus proliferation is usually obvious within one week of culture. Two types of callus can be formed in this system which are distinguishable by their respective sites of origin. One occurs when the entire embryo flips over because of continued embryonic development. The callus which develops directly from the embryonic axis is termed EMBRYONIC CALLUS. The other type occurs when embryonic growth (apparently) ceases, and the embryonic axis remains in contact with the medium. Here callus proliferates directly from the dome of the scutellum and is termed SCUTELLAR CALLUS (Rafaill 1979). It has been reported that the relationship between the age, size, and genotype of the embryo and the concentration of the hormone affects the type of callus produced (Green and Phillips 1975; Rafaill 1979). Such a study is relatively easy to reproduce for a classroom experiment. One approach would be to have one group of students excise embryos of a given age and place them on medium containing, for example, 1.0 mg/l 2,4-D, while a second group excises embryos of the same age on medium containing a different concentration of hormone. By carefully organizing the experiment it becomes easy to cover a range of ages (and therefore sizes) and hormone concentrations. An example of data collected from such an experiment is given in Tables 3.1 and 3.2. One obvious conclusion from such a study is that the ability to produce scutellar versus embryonic callus is influenced by the age, size, and genotype of the embryo as well as by the concentration of hormone utilized to initiate the tissue. For each genotype there is apparently a different combination of factors required to initiate scutellar versus embryonic callus. Students should be asked to speculate on what the relationship between these factors might be. Some ideas may be found in Aitchison et al. (1977).

Table 3.1. Ability of immature embryos to form callus.

Genotype	Embryo Age[1] (d)	Embryo Size[2] (mm)	Number Isolated	Embryonic Callus[3]	Scutellar Callus[4]
genetic stock[5]	14	3–4	22	20	0
genetic stock	12	2–3	34	12	14
A188[6]	16	5	20	20	0
A188	12	1.5–2.5	4	2	0

(1) Age refers to number of days after pollination when the embryo was excised.
(2) Size is the length of the embryo.
(3) Embryonic callus: embryo flipped over because of continued embryonic growth; callus formed from the developing embryonic structures.
(4) Scutellar callus: embryo did not flip over; embryonic growth aborted; callus formed from the dome of the scutellum.
(5) Represents the F₁ of M14/W23 by a **p r y** strain derived from a synthetic involving Longfellow Flint, inbred Kys, and Argentine Pop.
(6) An inbred strain.

Table 3.2. Influence of hormone concentration on callus production.
All embryos were from the genetic stock, were 12 days old when excised,
and measured 2–3 mm in length.

Hormone Conc (mg/l)	Number Isolated	Embryonic Callus	Scutellar Callus	Percent Embryonic	Percent Scutellar
0.5	36	12	14	33.3	38.9
1.0	34	12	14	35.3	41.2
2.0	26	12	5	46.2	19.2

Culture Maintenance and the Potential for Regeneration

A compact yellow callus proliferates once the remains of the embryo are removed after the first subculture. A few roots are produced which rapidly increase in number if the callus is left on the same medium without subculture for more than four weeks. Quite often areas of the callus will begin to green, and structures without any apparent organization appear in association with these green areas. If these cultures are subcultured to media containing the same concentration of 2,4-D the structures persist with the green areas and the callus continues to develop. If the cultures are instead transferred to media containing a reduced level of hormone (for example, less than 1.0 mg/l) the green areas sometimes persist, and roots begin to differentiate. This response will continue if the culture is transferred to media lacking hormone; a concomitant increase in the number of roots is usually observed. When cultures containing a plethora of roots are broken up and returned to media containing 1.0 mg/l or more of 2,4-D callus sometimes develops again, but at a much slower rate. The protocol to attempt the regeneration of plants from callus has been described by Green and Phillips (1975). Callus exhibiting various degrees of organization (presence of roots, leaves, "green areas", "structures

without any apparent organization", etc.) and believed to be totipotent is transferred to media containing 0.25 mg/l 2,4-D and then, three to four weeks later, to hormone-free media. Students wishing to pursue experiments in this area should be reminded that all cells contain the same quantity and quality of DNA, and that differentiation involves a selective "turning on" and "turning off" of genes. Thus, all cells are potentially totipotent if the correct combination of factors can be applied. Students should be asked to speculate on the effect of exogenously supplied hormones on the ability of cells (callus) to differentiate and dedifferentiate. Experiments (Green and Phillips 1975; Rafaill 1979) have suggested that only scutellar callus is totipotent. Thus, the student could consider, (1) what combination of factors in the development of scutellar callus "preserves" totipotency, or (2) what combination of factors in the development of embryonic callus precludes totipotency? (These questions are "thought questions" without specific answers. Ideas for discussion can be found in any of the references listed.) Further, maize has been a very difficult species to regenerate from culture, while a number of other species (carrots, tobacco, tomato) have proven relatively easy to regenerate. Students should speculate on what inherent factors may dictate whether regeneration will be easy or difficult.

Amino Acid Studies

Combinations of amino acids can be added to the basal medium in order to determine their effects on the growth of previously established callus. They can be grouped into "families" according to the metabolic pathways involved in their synthesis (Meister 1965; Miflin 1973; see Table 3.3), and experiments can then concentrate on one amino acid family at a time. It is not unexpected that certain combinations of amino acids should affect the growth of callus tissue. Inhibition of growth could easily result from feedback within a family (Green and Phillips 1974; Widholm 1974) or from cross reactions which inhibit the synthesis of an amino acid in another family (Miflin 1973). An exogenous supply of the inhibited product(s) should negate any suppression of growth, resulting in growth equal to or beyond the control level. Consequently, this experimental approach can be instructive in presenting the concept of the regulation of metabolic pathways. Although there are probably many ways to design such experiments, each with merit, one simple approach is described below. One must remember that the level of asparagine is relatively high (1.98 g/l = 15 mM) in the basal medium. Thus any experimental procedure must take this into account. However, since it has been established that the presence of asparagine has little, if any, effect on the growth of previously established callus cultures initiated from immature embryos (Green, personal communication; Rafaill, unpublished), it is suggested that for the amino acid experiments the basal medium be used without the addition of asparagine. (Whether or not asparagine is necessary for the initiation of callus from immature embryos has not yet been tested).

Table 3.3. Amino Acid Families.

Family	Amino Acids	Common Precursors
aromatic	phenylalanine **tryptophan** tyrosine	phosphoenolpyruvate & erythrose-4-phosphate
aspartate	asparagine aspartate isoleucine lysine methionine threonine	oxaloacetic acid
glutamate	arginine **glutamate** glutamine proline	α-ketoglutaric acid
histidine	histidine	phosphoribosyl pyrosphosphate
pyruvate	alanine leucine valine	pyruvic acid
serine	cysteine **glycine** serine	3-phosphoglyceric acid

Amino Acid Studies—Procedure

All amino acids, except tyrosine, should be maintained as stock solutions using double-distilled water as the solvent. The solutions should be refrigerated, and fresh solutions of each prepared monthly. Tryptophan can be maintained as a 2.0 mM solution, aspartic acid as a 5.0 mM solution, and the remaining amino acids as 10.0 mM solutions. Boiling water is required to dissolve tryptophan and aspartic acid. Tyrosine, because of its low solubility in water, must be added directly to the medium. Each experiment will consist of callus placed on two different media. One will simply be the basal medium, and the other will be the basal medium plus the amino acids being tested for their effect on the growth of callus. Each experiment involves the addition of the entire family of amino acids to the basal medium so that the final concentration of each is one millimolar. Further, each experiment involves 10 replicates for each media combination. To determine callus weights individual pieces of tissue are aseptically transferred to sterile petri dishes and weighed; the actual fresh weight of the tissue is determined by taring. The effect on callus development is determined by comparing the final fresh weight of a

Table 3.4. Effect of the asparate group on callus growth expressed as "fold fresh weight increase." Concentration of each amino acid was 1 mM in the experimental cultures, with 1.0 mg/1 2,4-D. Mean is presented ± the 95% confidence limit. The limit was calculated using t values.

	Control	Experimental
	1.7602	2.5579
	1.6901	2.1387
	1.3990	2.9167
	3.6812	4.2289
	4.6934	2.1322
	1.8846	1.7391
	2.1728	3.0493
	9.5317	
$\bar{x}$ ± confidence limit	3.3 ± 2.30	2.7 ± 0.76

culture after 28 days to its initial fresh weight. The difference in these weights, expressed as the "fold fresh weight increase" (Sternheimer 1954), is the ratio of the final fresh weight to the initial fresh weight. A sample of the kind of data obtained in this type of experiment is presented in Table 3.4.

Carrots

Culture Initiation

Seeds are surface-sterilized in the same manner as with the maize embryos. The seeds are then aseptically transferred to "germination chambers"; these consist of sterile petri dishes containing a piece of sterile filter paper thoroughly moistened with sterile distilled water. The chambers are then placed in the dark to allow the seeds to germinate. The root and petiole should reach a length of 2–3 cm within a week to ten days, depending upon temperature. Germination can be hastened if the chambers are incubated; however, they are then much more susceptible to dessication. Do not allow the filter paper to dry! Aseptically section the root or petiole into 0.5 cm pieces and place three to five of these on the carrot medium in suitable culture vessels. Callus is initiated best on low levels of 2,4-D (less than 1.0 mg/1). Incubate in the dark at 26–28°C. After three to four weeks the callus should be about five times the size of the original explant. Several questions can be asked concerning the ability to initiate carrot callus. One could involve a comparison of the ability of root versus petiole sections to form callus. A second approach might involve a comparison of the efficiency of callus initiation of different portions of a given root or petiole section (for example, root tip versus a more distal section). A third consideration might be to examine callus initiation from the same section (for example, a petiole tip) cut from different seeds over a range of days (that is, 3-day-old petiole tips, 4-day-old petiole tips, etc.). A fourth
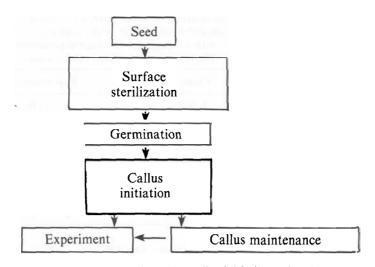
Figure 3.1. Procedures for maize callus initiation and maintenance.

approach might involve an examination of callus initiation from different varieties of carrot, and a fifth could involve callus initiation over a range of hormone concentrations. Data from these kinds of experiments can be organized in a fashion similar to that presented in Table 3.1. Generally speaking, callus initiation from carrot is relatively easy compared to callus initiation from maize, and, consequently, students might find carrot a much more rewarding experience for their first exposure to plant tissue culture.

Callus Maintenance and the Potential for Regeneration

Callus is transferred to carrot medium containing the same level of hormone as was used to initiate the culture, and is incubated at 28–30°C with a 16:8 L:D photoperiod. In appearance carrot callus seems to grow much faster and is less apt to produce excessive amounts of roots or "structures without any apparent organization". In fact, it is not uncommon to find carrot callus spontaneously regenerating, as evidenced by the production of many leaflets. Thus an obvious difference between maize and carrot callus is that the former tends to spontaneously produce roots and is difficult to regenerate, while the latter spontaneously produces leaflets and is easy to regenerate (following the regeneration protocol described for maize). Students should be asked to suggest reasons for this. In doing so, they should be reminded that maize is a monocot while carrot is a dicot.

Amino-Acid and Other Studies

To perform amino-acid experiments with carrot, follow the procedures outlined for maize.
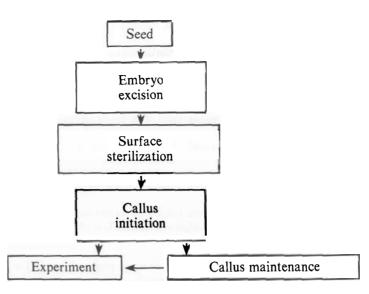
Figure 3.2. Procedure for carrot callus initiation and maintenance.

Other Experiments

A number of other experiments can be performed with tissue culture systems, such as the isolation of plant protoplasts, chromosomal staining, and preparing the tissues for histological study. It is suggested that Gamborg and Wetter's text (1975) be consulted as a primary source for further study.

Supplies and Materials

2% solution of sodium hypochlorite
70% ethanol
Analytical balance
Autoclave
Carrot seeds (any variety)
Double-distilled water
Filter paper
Forceps (tissue type)
Fresh ears of corn
Growth chamber, 28–30°C, 16:8 L:D
   photoperiod

Incubator, 26–28°C
Scalpels
Supply of 100–150 ml beakers
Supply of 125 ml flasks
Supply of petri dishes
Transfer hood (although a laminar-flow
   type hood is preferable, a tissue
   culture hood, Labonco 11000, is
   adequate)

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APPENDIX A
Plant Tissue Culture Terminology (adapted from Street 1977)

PLANT TISSUE CULTURE is a blanket term encompassing the culture of: seedlings or larger plants (PLANT CULTURES); isolated mature or immature embryos (EMBRYO CULTURES); isolated plant organs such as root tips or leaf primordia (ORGAN CULTURES); tissues arising by proliferation from segments (explants) of plant organs (TISSUE OR CALLUS CULTURES); isolated cells or very small aggregates of cells dispersed in liquid media (SUSPENSION CULTURES).

The term CALLUS CULTURE was (probably) originally derived from the fact that the proliferation of such material is induced by injury during the process of excising the explant. Most now agree that this is only part of the story since the removal of the explant from the controls imposed upon its tissues by the whole plant and the provision to the explant of appropriate nutrients and growth-regulating substances may be equally important to proliferation. Some prefer to describe such cultures as TISSUE CULTURES since they can be derived from different tissues in the parent explant and can differ very markedly from one another. In either case such cultures always contain both dividing and non-dividing cells and the latter may consist of several distinct cell types within the tissue or callus mass. Thus, the term tissue or callus culture can be appropriately applied to any culture growing on solid medium and consisting of many cells in protoplasmic continuity. It does not imply any structural or functional homogeneity of the constituent cells or equivalence with any normal plant tissue. The term SUSPENSION CULTURE is self-explanatory referring to cells and cell aggregates growing dispersed in a moving liquid medium. The use of the term ASEPTIC is preferable to sterile when referring to the above cultures since they are grown from surface-sterilized explants under aseptic conditions (conditions designed to exclude contaminants). However, it cannot be claimed that at all times the cultures are free from contaminating organisms.

APPENDIX B
Culture Media

Maize Medium

A complete description of the maize medium is presented in Table 3.5. The stock solutions are prepared using double-distilled water and refrigerated. The organic solution (G) is freshly prepared monthly, while fresh mixtures of the other stock solutions are prepared only as needed. Stock solution F requires special preparation. This is accomplished by separately dissolving the $FeSO_4 \cdot 7H_2O$ and the $Na_2 \cdot EDTA$ in about 200 ml of water. The EDTA solution is then heated and mixed, while stirring, with the iron solution. After cooling the volume is adjusted to one liter. Heating and stirring result in a more stable iron-EDTA complex. All other stock solutions are made simply by sequentially mixing, while stirring, each ingredient in a volume of water and then adjusting to one liter. The 2,4-dichlorophenoxyacetic acid (2,4-D) is maintained as a 2.2 mM stock solution. This is prepared by dissolving 50 mg of 2,4-D in 5 ml of ethanol, heating slightly, and then gradually diluting to 100 ml with double-distilled water. This solution is refrigerated and a fresh mixture is prepared monthly. The medium is prepared by first dissolving the asparagine in about 200 ml of hot, double-distilled water. The volume is diluted to about 800 ml and then, in order, the sucrose, stock solutions, and hormone are added while stirring. The final volume is corrected to one liter, the pH adjusted, and then the agar added. The resultant mixture is then heated, while stirring, to boiling to dissolve the agar. The medium is then dispensed into the desired culture vessels and then autoclaved for 20 minutes at 121°C.

Table 3.5. Maize tissue culture medium. Rafaill (1979).

Stock Solution	Components	gm/l
A (20 ml/l)	NH_4NO_3	82.5
B (20 ml/l)	KNO_3	95.0
C (5 ml/l)	H_3BO_3	1.24
	KH_2PO_4	34.0
	KI	0.166
	$Na_2MoO_4 \cdot 2H_2O$	0.05
	$CoCl_2 \cdot 2H_2O$	0.005
D (5 ml/l)	$CaCl_2 \cdot 2H_2O$	88.0
E (5 ml/l)	**$MgSO_4 \cdot H_2O$**	74.0
	$MnSO_2 \cdot H_2O$	3.38
	$ZnSO_4 \cdot 7H_2O$	1.72
	$CuSO_4 \cdot 5H_2O$	0.005
F (5 ml/l)	$Na_2 \cdot EDTA$	7.45
	$FeSO_2 \cdot 7H_2O$	5.57
G (1 ml/l)	Glycine	7.7
	Niacin	1.3
	Thiamine-HCl	0.25
	Pyridoxine-HCl	0.25
	Ca-panthothenate	0.25
addenda:	L-asparagine	1.98 g/l
	Sucrose	20.0 g/l
	Agar (Difco)	8.0 g/l
hormone:	2,4-D	0.25–4.0 mg/l
pH:	**Adjust to 6.0 with NaOH**	

Carrot Medium

A complete description of the carrot medium is presented in Table 3.6. The stock solutions are prepared in the same manner as above. Solution D must be stored in a brown bottle. To prepare this medium, each of the items is added, in order, to double-distilled water while stirring. The volume is adjusted to one liter, the pH adjusted, and the agar added. After heating to dissolve the agar as above, the medium is dispensed into the appropriate culture vessels and autoclaved for 20 minutes at 121°C.

Table 3.6. Medium for carrot tissue culture.
(Adapted from Gamborg and Wetter 1975).

Stock Solution	Components	gm/l
A (1.0 ml/l)	$MnSO_4 \cdot H_2O$	1.0
	H_3BO_3	0.3
	$ZnSO_4 \cdot 7H_2O$	0.2
	$Na_2MoO_4 \cdot 2H_2O$	0.025
	$CuSO_4 \cdot 5H_2O$	0.0025
	$CoCl_2 \cdot 6H_2O$	0.0025
B (1.0 ml/l)	Niacin	0.1
	Thiamine-HCl	1.0
	Pyridoxine-HCl	0.1
	Myo-inositol	10.0
D (1.0 ml/l)	KI	0.075
addenda:	$NaH_2PO_4 \cdot H_2O$	150 mg/l
	$(NH_4)_2SO_4$	134 mg/l
	$MgSO_4 \cdot 7H_2O$	250 mg/l
	Sucrose	20 g/l
	Maize soln B	26.4 ml/l
	Maize soln F	1.5 ml/l
	Maize soln C	1.7 ml/l
	Agar (Difco)	6.0 g/l
hormone:	2,4-D	0.1–1.0 mg/l
pH:	Adjust to 5.5 with NaOH	