

Chapter 9

Plant Tissue Culture Techniques

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Introduction

Plant tissue culture techniques are essential to many types of academic inquiry, as well as to many applied aspects of plant science. In the past, plant tissue culture techniques have been used in academic investigations of totipotency and the roles of hormones in cytodifferentiation and organogenesis. Currently, tissue-cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture. For example, select plants can be cloned and cultured as suspended cells from which plant products can be harvested. In addition, the management of genetically engineered cells to form transgenic whole plants requires tissue culture procedures; tissue culture methods are also required in the formation of somatic haploid embryos from which homozygous plants can be generated. Thus, tissue culture techniques have been, and still are, prominent in academic and applied plant science.

The techniques demonstrated in these exercises range from simple ones that can easily be performed by beginning students to those done by botany or physiology students. Experiment 1 and 2 employ plant material derived from aseptic seed germinations, while Experiments 3, 4, and 5 use portions of large intact plants. Experiment 1 demonstrates "in vitro" morphogenesis and totipotency and has been used successfully by beginning classes containing both biology majors and non-majors (expected results are presented in Appendix A). The remaining experiments are designed for use by more advanced students.

For further reading see Bottino (1981), Butcher and Ingram (1976), Dodds and Roberts (1985), Street (1973), Sunderland and Roberts (1977), and Wetherell (1982).

Terminology

Aseptic	Free from microorganisms
Callus plant hormone levels.	Undifferentiated, swollen cell mass forming under the influence of elevated
Etiolation	Yellow and stretched plant; parts elongate until light is intercepted.
Explant	Part of an organism used in "in vitro" culture.

IAA	Indoleacetic acid; a plant hormone increasing cell elongation and, under certain circumstances, implicated in stimulating cell division and root formation. IAA moves in a polar manner in plants forming an IAA gradient in tissues. Orientation of plant organs, then, influence callus formation and morphogenesis.
"in vitro"	"In glass"; as in tissue culture methods
Morphogenesis	Change in shape
Polarity	Orientation in gravitational field.
Primordia	The earliest detectable stage of an organ, such as a leaf, root or root branch.
Root hairs	Epidermal cell extensions of young root that increase absorptive surface area.
Totipotency	The establishment of missing plant organs or parts; formation of a whole plant from a few cells or small portion of a plant.
Wound response	Formation of callus in wounded area.

Laboratory Requirements for Tissue Culture

General Organization

Localize each portion of the tissue culture procedure in a specified place in the laboratory. An assembly-line arrangement of work areas (such as, media preparation, glassware washing, sterilization, microscopy, and aseptic transfers) facilitates all operations and enhances cleanliness. Media (tissue culture and nutrient agar) are available from Carolina Biological Supply Co., Burlington, NC. Laminar flow hoods are available from several suppliers.

Glassware

Use glassware that has only been used for tissue culture and not other experiments. Toxic metal ions absorbed on glassware can be especially troublesome. Wash glassware with laboratory detergent, then rinse several times with tap water and, finally, rinse with purified water.

High-purity Water

Use only high-purity water in tissue culture procedures. Double glass distilled water or deionized water from an ion-exchanger are acceptable. Water should not be stored, but used immediately. Regular maintenance and monitoring of water purification equipment are necessary. Purified water for tissue culture can also be purchased.

Plant Material

Plants used in tissue culture need to be healthy and actively growing. Stressed plants, particularly water-stressed plants, usually do not grow as tissue cultures. Insect and disease-free greenhouse plants are rendered aseptic more readily, so contamination rate is lower when these plants are used in tissue culture procedures. Seeds that can be easily surface sterilized usually produce contamination-free plants that can be grown under clean greenhouse conditions for later experimental use.

Aseptic Technique

The essence of aseptic technique is the exclusion of invading microorganisms during experimental procedures. If sterile tissues are available, then the exclusion of microorganisms is accomplished by using sterile instruments and culture media concurrently with standard bacteriological transfer procedures to avoid extraneous contamination.

Media and apparatus are rendered sterile by autoclaving at 15 lbs/inch² (121°C) for 15 minutes. The use of disposable sterile plasticware reduces the need for some autoclaving. Alternative sterilization techniques such as filter sterilization must be employed for heat-labile substances like cytokinins.

Aseptic transfers can be made on the laboratory bench top by using standard bacteriological techniques (i.e., flaming instruments prior to use and flaming the opening of receiving vessels prior to transfer). Aseptic transfers are more easily performed in a transfer chamber such as a laminar flow hood, which is also preferably equipped with a bunsen burner (Bottino, 1981).

If experimental tissues are not aseptic, then surface sterilization procedures specific to the tissues are employed. Common sterilants are ethyl alcohol and/or chlorox with an added surfactant. Concentration of sterilants and exposure time are determined empirically.

Experiment 1: Demonstration of "in vitro" Morphogenesis and Totipotency of Seedling Explants

A simple exercise demonstrating plant totipotency as well as the nutritional requirements of different plant organs employs shoot tip and root tip explants cut from aseptically germinated seedlings. Each type of explant (excised part of the intact organism) is transferred to three simple tissue culture media.

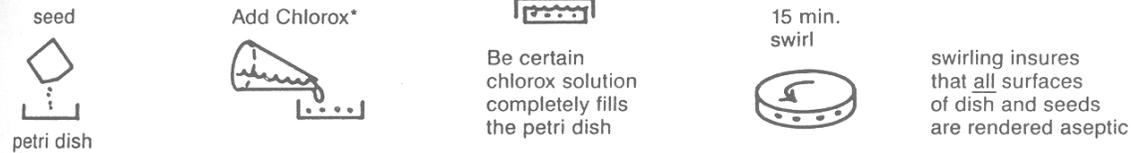
Background

During seed formation, the developing embryo and associated tissues tend to exclude pathogens and foreign materials that may be in the parent plant. Contents of the seed, then, are essentially aseptic and the resultant seedlings can be maintained in the aseptic condition if the outer surface of the seed (seed coat) is sterilized with sodium hypochlorite (or other surface sterilant) prior to germinating the seeds in a sterile petri dish.

Methods: Week 1

The manipulations that are required for the germination of aseptic seedlings are outlined below and illustrated in Figure 9.1.

OUTSIDE OF TRANSFER CHAMBER:



CHAMBER PREPARATION:

Be certain that chamber has been aseptically cleaned prior to use. First, swab the floor and walls of chamber with 70% ethanol. Turn on + run Laminar hood for 10-15 minutes. Prior to the next aseptic transfers:

1. roll up sleeves
2. wash hands with soap
3. wipe external aspects of petri dish with 70% ethanol
4. wipe hands with 70% ethanol

INSIDE TRANSFER CHAMBER:

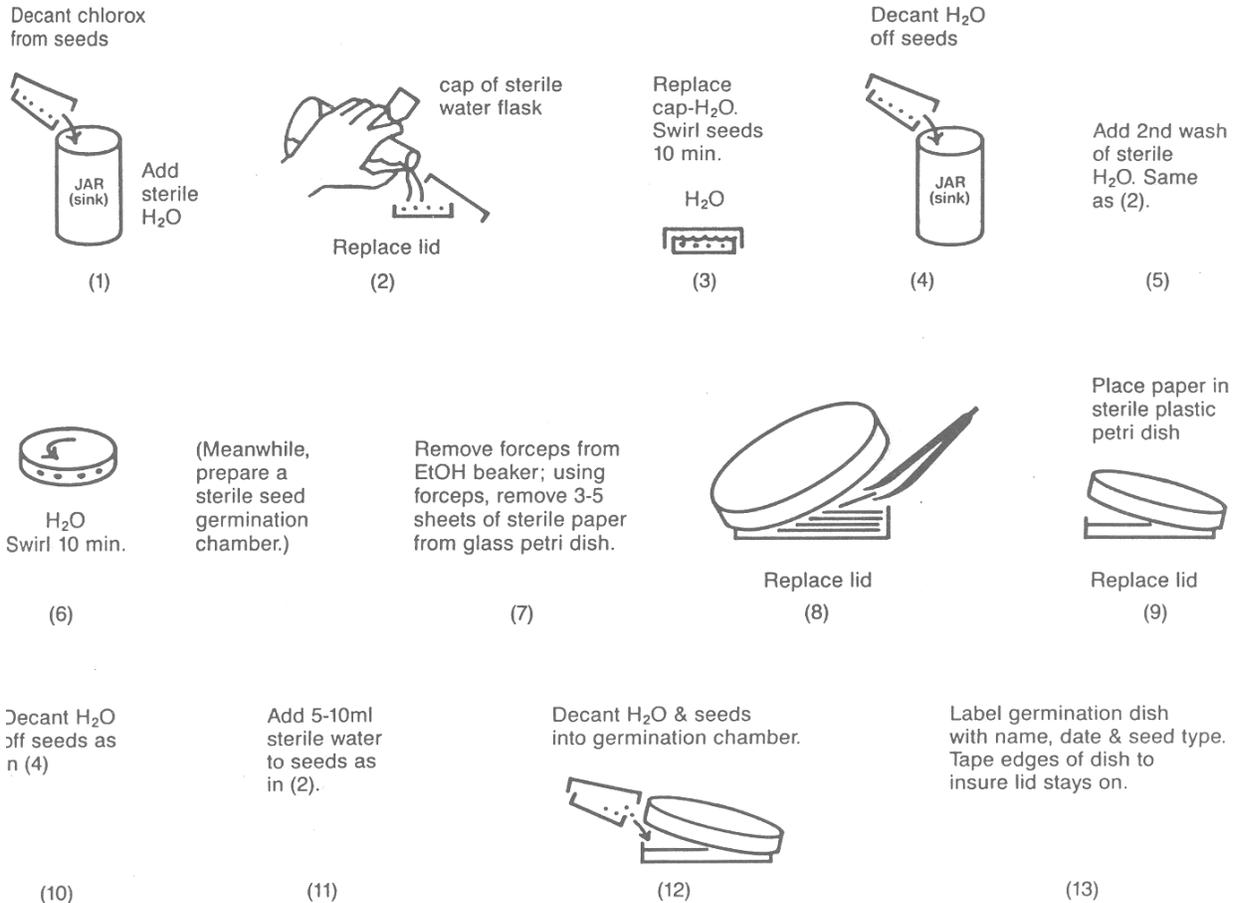


Figure 9.1. The manipulations required for the germination of aseptic seedlings (Experiment 1, Methods: Week 1).

1. *Outside the laminar flow hood:* Place several (5 to 10) tomato or lettuce seeds in a small petri dish. Fill the petri dish with a 7% chlorox solution to which a drop of wetting agent has been added. The soapy chlorox solution is usually a good surface sterilant. Swirl the seeds intermittently during the 10- or 15-minute chlorox treatment.
2. *Preparation for aseptic transfers:* Begin by washing your hands and forearms with soap, followed by swabbing with 70% ethyl alcohol (EtOH). Sterilize the laminar flow hood by wiping the inside (top, sides, and bottom) with EtOH. Turn on the hood; 10–15 minute operation of the hood before use insures aseptic conditions within the work area of the hood. Continue to swirl the seeds intermittently during the chlorox treatment. Prior to actual aseptic transfers inside the chamber, swab hands and forearms with EtOH again; also wipe the external surface of the petri dish before placing it inside the hood. The hood should contain the following: a large jar which can be used as a "sink," flasks of sterile water, forceps in a beaker of ethanol, sterile filter paper (5–7 cm diameter filter paper can be sterilized in glass petri dishes), and sterile petri dishes which can be used as the seed germination dishes.
3. *Inside the laminar flow hood:* Decant chlorox and replace with sterile H₂O. Rinse this way twice. Each rinse should rest 10 minutes. Prepare the sterile germinating petri dish by retrieving a forceps from the 70% EtOH beaker. Using the sterile forceps remove three (3) rounds of sterile filter paper from a sterile container and place them in the germinating dish (sterile plastic petri dish). Finally, add 5–10 ml of sterile H₂O to the seeds; decant seeds and water into the sterile germinating dish and incubate at 25°C until the next laboratory. (Both tomato and light-insensitive lettuce seeds germinate in the light. Since shoots become green but roots remain white under these conditions, seedling morphology is recognized more easily when light-germinated.)

Methods: Week 2

Examine the contents of the aseptic germinating dish *without* opening the lid. If there is no fungal or bacterial contamination around the seedlings, proceed; if contamination exists, request a dish of aseptic seedlings from the instructor. The manipulation required for the transfer of seedling explants to Mineral Salts (M) and Minimal Organic (O) growth media are outlined below and illustrated in Figure 9.2.

1. Swab chamber, hands, and upper/lower surfaces of petri dish with 70% ethanol.
2. Place germinating dish in transfer chamber.
3. Remove scalpel or scissors from the ETOH beaker already in the hood. Slip instrument between sheets of sterile toweling to remove ETOH (ethanol).
4. Lift one edge of lid and cut off no more than 10 mm of root tip. Excise two root tips. Lower lid. Place scalpel back into ETOH beaker.
5. Place tubes with sterile media into the transfer chamber. (Media formulae are given in Appendix B.) Use one tube of Minimal Organic Medium (O) and one of Mineral Salts Medium (M). Loosen these caps.

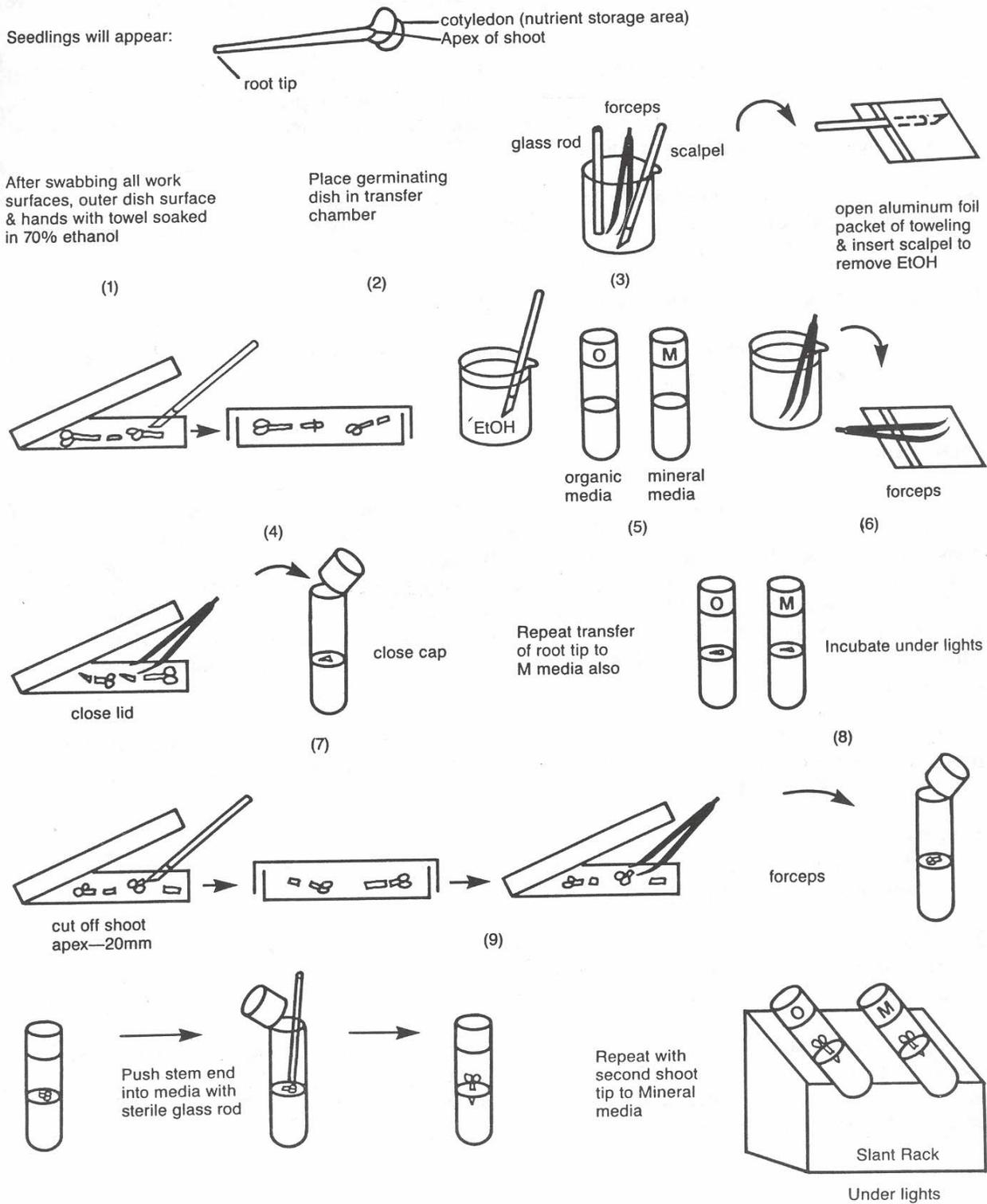


Figure 9.2 The manipulations required for the transfer of seedling explants to Mineral Salts (M) and Minimal Organic (O) growth media (Experiment 1, Methods: Week 2).

6. Remove forceps or inoculating loop from ETOH. Slip between sterile toweling to remove ETOH.
7. Remove excised root tip from germinating dish and transfer to the surface of the Minimal Organic Medium (O). Transfer second root tip to the surface of the Mineral Salts Medium (M). Caution: pick up root tip by the severed end; damage to the apical meristem disrupts mitosis! Measure or estimate length of root tips. Record.
8. Using aseptic technique as above, prepare and transfer one shoot tip into each type of media. Pick up the shoot tip by the severed end and insert it part way into the medium with an overall vertical orientation of the cotyledons and shoot tip. Record size and shape of shoot tip.
9. Place the four tubes in a slant rack under lights.
10. Examine cultures each week. Record observations on the amount of growth and morphogenesis of both root and shoot cultures.

Observations

As cultures progress it should be possible to correlate size/shape changes with the nutrient content of the medium. A third medium, the B-deficient Medium, contains the same mineral constituents as does the Mineral Salts Medium (M) and the same amount of sucrose as the Minimal Organic Medium (O), but is devoid of B vitamins. Thus, this medium is referred to as B-deficient (-B). Shoot tips and root tips have been transferred to this demonstration medium. Growth on this medium can be evaluated and compared with growth on student experimental media M and O. The Mineral Salts Medium (M) is the basal growth medium, supplying essential mineral nutrients for autotrophic plant growth (Appendix C).

Predict the resultant growth in each circumstance, then monitor the growth and development of root and shoot explants in each medium (M, O, and -B) and evaluate the following (record observations in Table 9.1):

1. Effect of B-vitamins on:
 - a) shoot growth (increase in size) and morphology (change of shape), and
 - b) root growth and morphology.
2. Effect of organic medium containing sucrose on:
 - a) shoot growth and morphology, and
 - b) root growth and morphology.

Optional: media M, O, and -B are set up with root tip and shoot tip explants in darkness. This set of samples can be observed along with those in the light to evaluate the effect of light as well as media contents on the growth and development of plant organs. A row labeled "etiolation" would be added to the bottom of Table 9.1.

Experimental observations should include the following:

1. Parameters: temperature; light quality, duration, and intensity.
2. Drawings to scale.
3. Gross measurements (length, biomass accumulation, extent of morphogenesis, totipotency, primordia, number of branches) after 1 week and 2 weeks.
4. *Net* changes (Table 9.1).
5. Does the irregular orientation of the shoot explant change the growth pattern? How can these observations be explained?

Table 9.1. Use blank table to record results from Experiment 1.

<i>Media</i>	<i>Shoot</i>			<i>Root</i>		
	Mineral	B-deficient	Organic	Mineral	B-deficient	Organic
<i>Cap color</i>	White	Red	Blue	White	Red	Blue
<i>Change in length</i>						
<i>Change in Biomass</i>						
<i>Extent of morphogenesis</i>						
<i>Totipotency</i>						
<i>Primordia</i>						
<i>Number of Branches</i>						

Questions

1. Are all portions of the seedling totipotent?
2. Is autotrophic and heterotrophic growth of different plant organs apparent?
3. How could the experimental design be changed to more completely evaluate B-vitamin and extra-sucrose effects?
4. If B vitamins seem important to root growth and development, how are vitamins probably supplied to plant roots in intact plants?

Experiment 2: Effects of Hormone Balance on Explant Growth and Morphogenesis

Background

Plant hormones, like animal hormones, are relatively small molecules that are effective at low tissue concentrations. The two types of plant hormones used in this experiment are cytokinins and auxins.

Cytokinins are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division (Ting, 1982). Cytokinins also produce a delayed response in undifferentiated tissue which is the formation of shoot primordia. Both naturally occurring cytokinins, such as zeatin and synthetic analogs, such as kinetin, demonstrate cytokinin effects (Figure 9.3). Although low tissue concentrations of cytokinins (e.g., 1×10^{-8} M zeatin) have noticeable effects, higher concentrations are found in actively dividing tissues such as those of plant embryos and developing fruits.

Auxins are indole or indole-like compounds that stimulate cell expansion, particularly cell elongation. Auxins also promote adventitious root development. Indoleacetic acid (IAA), a naturally occurring auxin, and naphthaleneacetic acid (NAA), a synthetic auxin, are depicted in Figure 9.3. Only small amounts of auxin (1×10^{-6} M) are required to demonstrate an IAA response and even smaller amounts of synthetic auxin (e.g., NAA) are required for a tissue response. The likely reason for potency of synthetic auxins is their stability in plant tissue (i.e., the enzymes and processes that degrade IAA do not "recognize" synthetic auxins). Synthetic auxins, then, are more effective hormones that also last for an extended length of time. Furthermore, light influences the physiological activity of IAA while synthetic auxins are not as light sensitive.

Plant hormones do not function in isolation within the plant body, but, instead, function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue. Since hormone balance is presumably important to the overall effect on growth and morphological changes, then the hormone differentials in each of the experimental media (A, B, and C) should produce somewhat different effects on the growth and development of excised explants.

Source of Aseptic Explant Material

During seed development the embryos are formed with a placenta-like interface of intervening tissues between parental vascular supply and the embryo proper. This circumstance depresses passive migration of most foreign bodies and microorganisms into the developing embryo. If the embryo which often develops aseptically is released from the seed by aseptic germination procedures, then aseptic seedlings result. Any part of the aseptic seedling can be used as "in vitro" experimental material. In this experiment three explant types will be used: *hypocotyl* (undeveloped lower stem), *epicotyl* (shoot apex), and *cotyledons*.

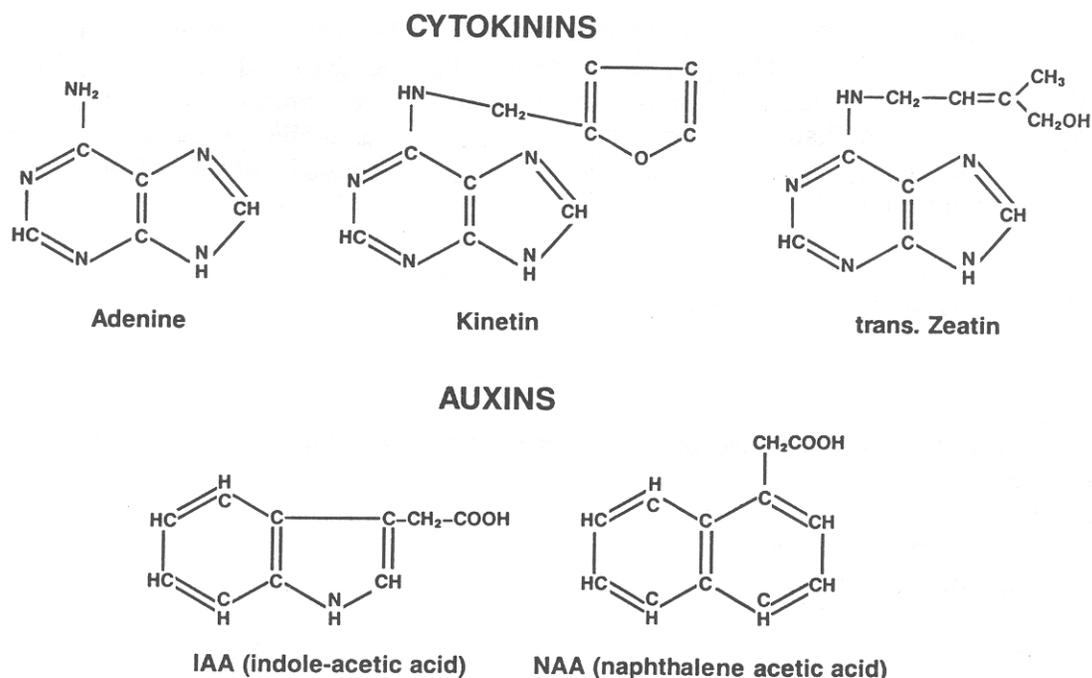


Figure 9.3. Structural formulae of plant growth and differentiation hormones used in Experiment 2.

Media Formulae

Media A, B, C, D, and E each contain the same complement of minerals, that is, salt base as in Medium D (Appendix D). The effect of minerals alone on explant growth and development constitute "basal growth rate" against which the effects of other media constituents can be measured. Medium D, then, serves as the base-line control for endogenous growth. Medium E, containing both essential minerals plus sucrose, constitutes the organic and inorganic control which can be used as the base-line indicator of explant growth when both minerals and sucrose are supplied. In addition to the substrate, sucrose, Medium E contains two organic growth factors, inositol and thiamine, which promote sugar metabolism and general anabolic growth processes. Medium E also contains additional phosphate thereby matching the phosphate concentrations of the experimental media (A, B, C). The experimental media contain similar inorganic and organic complements, but differ in hormone content.

Since cytokinins are derived from adenine, adenine sulfate has been added to each of the experimental media (A, B, C). In addition, either kinetin or 2iP ([2-isopentenyl]-adenine), both of which are synthetic cytokinins having immediate hormone activity, are supplemental cytokinins in media A, B, and C. Of the three experimental media, Medium A contains the highest amount of active cytokinin (30 mg/liter), while media B and C contain much lower amounts (2 mg/liter and 1 mg/liter, respectively).

Conversely, Medium A contains only a small amount of auxin (0.3 mg IAA/liter), while Medium B contains a higher amount (2 mg IAA/liter). Medium C contains the lowest absolute concentration of auxin (0.1 mg NAA/liter), but this synthetic auxin is more efficient in promoting cell expansion and root formation than the naturally occurring auxin, IAA. Medium C, then, may actually represent the formula with the highest physiological auxin activity.

Since cytokinin/auxin balance is reportedly important to the final overall effect on growth and development, the results for each experimental media may be expected to differ. The balance

represented by Medium A is decidedly skewed towards a high cytokinin/low auxin ratio. Medium B represents a more even distribution of cytokinin and auxin, while Medium C may have an effectively higher auxin than cytokinin ratio because of the "in vivo" stability of NAA as well as its effectiveness as an auxin.

Methods: Week 1 (Aseptic Seed Germination)

Materials:

cucumber seeds (tomato may also be used; sterilization procedure, Experiment 1)
95% ethanol
sterile jars, sterile water
25% chlorox, freshly prepared
sterile forceps or spatula

Procedure:

1. Sterilize seeds (cucumber) for 1 minute with 95% EtOH.
2. Rinse in sterile water
3. Sterilize in 25% bleach for 5 minutes and rinse three times with sterile water.
4. Transfer 10 seeds with a sterile forceps or spatula to a nutrient agar plate.
5. Incubate for 1 week (20–23°C).

Methods: Week 2

Seedling explants (approximately 1 cm in length) of aseptically germinated cucumber (or tomato) can be cut from the seedlings as shown in Figure 9.4. Aseptic techniques including the use of the laminar flow hood are necessary to evaluate growth experiments in which nutrient rich media are used. An explant (hypocotyl, epicotyl, or cotyledon) should be placed on each of the following *experimental* media:

- A) Murashige Shoot Multiplication, Medium A,
- B) Murashige Shoot Multiplication, Medium B, and
- C) Murashige Shoot Multiplication, Medium C,

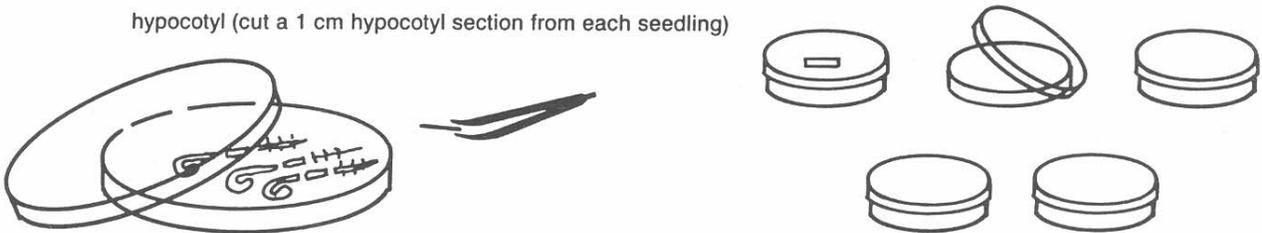
which contain different concentrations of growth hormones (Appendix D). For comparison, one explant of each type should be placed on each *control* media:

- D) Murashige and Skoog Salt Base, Medium D, and
- E) Murashige Minimal Organic Medium + Sucrose with $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, Medium E.

Seal the petri dishes with parafilm to prevent desiccation and incubate at low light intensity until next week. Record incubation conditions.



1. Remove lid of germination dish and cut explants from 5 seedlings as shown below. Cover the dish and replace the instrument in EtOH.



2. Remove the forceps from EtOH. Slip between sterile toweling to remove EtOH. Remove each excised hypocotyl from the germination dish to one of the 5 media plates. Slightly depress the hypocotyl into the media for adequate transfer of nutrients into the explant (piece of hypocotyl).



3. Repeat with epicotyl explants using one of each of 5 media and cotyledon explants using one of each of 5 media. A total of 15 plates (3 explant types on 5 media types) results for analysis in this factorial experiment.

4. Seal the plates with parafilm and incubate under low light intensity until next week. Record incubation conditions (temp., light cond.).

Figure 9.4. The transfer of explants to differential growth media using aseptic techniques (Experiment 2, Methods: Week 2).

Discussion Questions

What effect does a hormone balance that is applied pharmacologically "in vitro" have on seedling explant growth and morphogenesis? Which media formulations produce callus? To what extent? On which explants? Were these results predictable? What media produce anomalous

growth rather than callus? How is this explained? Are adventitious roots formed on all explants in Medium C? Which explants are readily totipotent on control media (D, E)? Is a wound response associated with observed totipotency? Does the hormone balance of experimental media (A, B, C) influence the extent of totipotency? Are the organs that form during organogenesis complete and apparently functional? Do microscopic observations of cell size, dimension, and orientation facilitate the interpretation of hormone effects? Do these explants grown in Medium A (high cytokinin, low auxin) show rapid cell division without cell expansion? Are shoot primordia forming on the explants in Medium A? Are callus cells disorganized and oversized?

Additional Studies

How does explant orientation in the medium influence results? If callused explants are placed on Medium A, will shoot primordia develop? Is it possible to generate an entire plant from a callused explant? Are serial transfers and the multiplication of callus possible? On which media? Is it possible to clone whole plants from multiplied callus? Under what conditions?

Experiment 3: Callus Formation and Multiplication

Callus is defined as an unorganized tissue mass growing on solid substrate. Callus forms naturally on plants in response to wounding, infestations, or at graft unions (Bottino, 1981). Since extensive callus formation can be induced by elevated hormone levels, tissue culture media designed to produce callus contain pharmacological additions of cytokinins and auxins.

Callus formation is central to many investigative and applied tissue culture procedures. Callus can be multiplied and later used to clone numerous whole plants. Additionally, various genetic engineering protocols employ callus initiation procedures after DNA has been inserted into cells; transgenic plants are then regenerated from transformed callus. In other protocols callus is generated for use in biotechnological procedures such as the formation of suspension cultures from which valuable plant products can be harvested.

Callus Formation

Explants from several parts of large intact plants can be used to form callus. The most successful explants are often young tissues of one or a few cell types. Pith cells of young stem are usually a good source of explant material. Initially, callus cells proliferate without differentiating, but eventually differentiation occurs within the tissue mass. Actively dividing cells are those uppermost and peripheral in the callus. The extent of overall differentiation usually depends on the hormone balance of the support medium and the physiological state of the tissue.

Callus Multiplication

Actively growing callus can be initiated on culture media with an even physiological balance of cytokinin and auxin (Tobacco Callus Initiation Medium; Appendix E). After callus biomass increases two to four times (after 2–4 weeks of growth), callus can be divided and placed on fresh Tobacco Callus Initiation Medium for callus multiplication. Multiplication procedures can be repeated several times (up to eight sequential transfers) before gross chromosome instability (or contamination) occurs.

Differentiation and Plant Regeneration

Multiplied callus can be stimulated to form shoots by increasing the cytokinin concentration and decreasing auxin content of culture media (Tobacco Shoot Development Medium; Appendix E). Shoot masses can be cut apart and transferred to rooting medium. Once rooted, regenerated plants can be acclimatized to natural rather than "in vitro" growth conditions. Regenerated plants are especially valuable if the parent plant was itself unique or if the plants were genetically engineered. If, for example, multiplied callus was first used to form suspension cultures on which genetic engineering or cell selection was accomplished, resultant regenerated plants via tissue culture could possess special traits or capabilities.

Materials and Methods

Callus Formation (Bottino, 1981)

1. Obtain a 5-cm section of tobacco stem.
2. Cut off all leaves.
3. Immerse it in a beaker of 95% ethanol for 15 seconds.
4. In the laminar flow hood, expose the pith by cutting away epidermis, cortex, and vascular tissue with a sterile scalpel (Figure 9.5).
5. Slice the exposed length of pith into a sterile petri dish.
6. Cover the dish to keep pith sterile.
7. Aseptically slice 5-mm cross-sections of pith.
8. Transfer one cross-section to each plate of Tobacco Callus Initiation Medium.
9. Cover the dishes, seal with parafilm, and place in an incubator at 22–25°C.

Callus Multiplication

1. Obtain a plate of tobacco callus.
2. Aseptically divide the callus into smaller pieces.
3. Transfer divided callus pieces to fresh Tobacco Callus Initiation Medium.

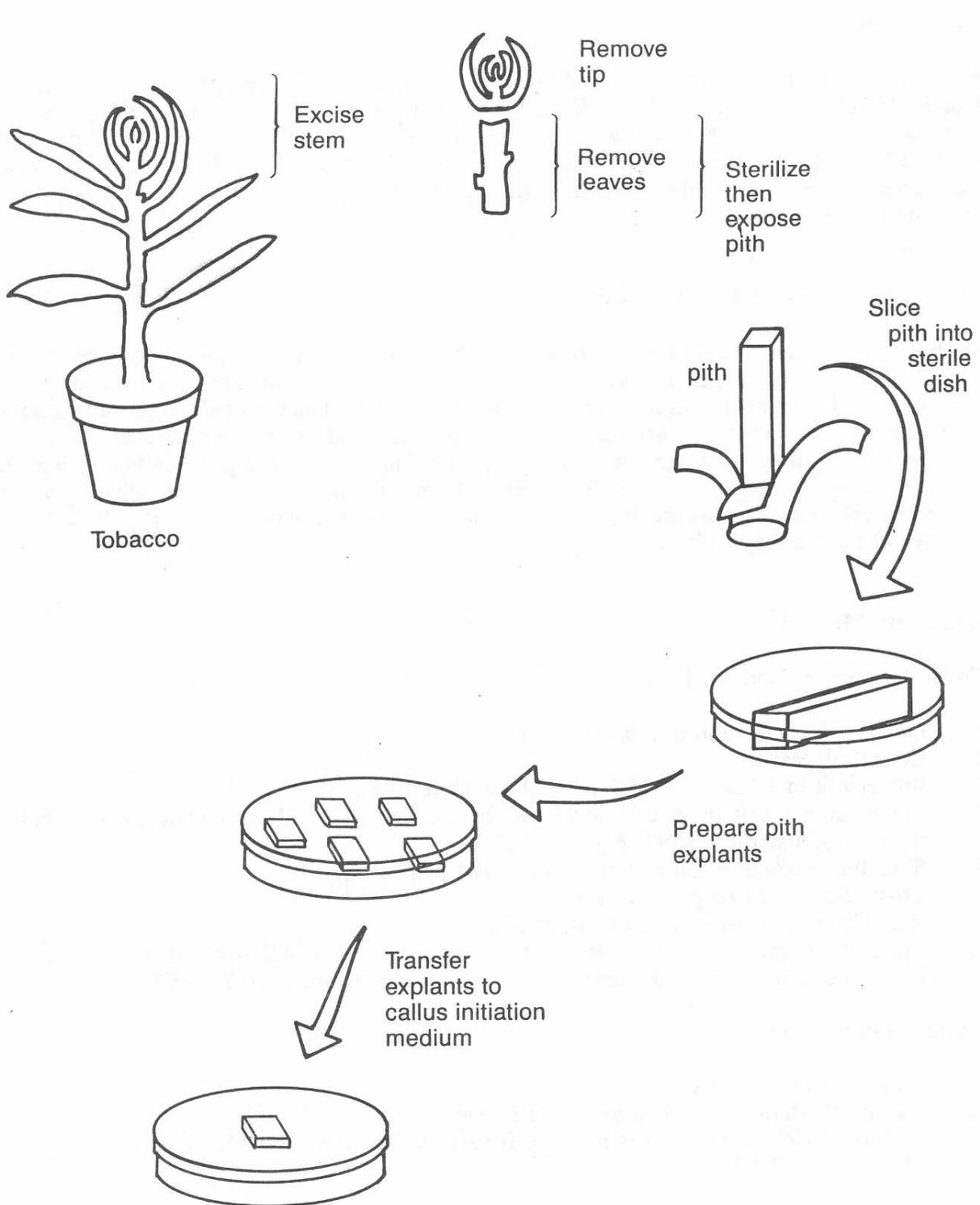


Figure 9.5. By using pith explant and plating procedures, callus can be generated from pith cells placed on Tobacco Callus Initiation Medium (Bottino, 1981).

Experiment 4: Establishment of Suspension Cultures

Background

Suspension cultures are suspensions of individual plant cells and small cell clusters (microcalli) grown in liquid media. Suspension cultures are established by transferring small pieces of callus to liquid medium which is subsequently placed on a gyratory shaker. Within a few days individual plant cells and microcalli should be detached from the original inoculum and growing in the constantly agitated medium. Suspension cultures grow best if the larger pieces of callus are removed after the culture has been initiated (Bottino, 1981).

Methods

1. Break up tobacco callus into small pieces and transfer to liquid medium (Tobacco Callus Initiation Medium, without the agar). Use 25–50 ml medium in 250-ml flask for adequate aeration for the swirling culture.
2. A thick suspension of callus should continue to proliferate within the next week on a gyratory shaker.
3. Remove large pieces of callus from suspension culture by passing through a sterile sieve. Large pieces of callus are usually detrimental to the maintenance of a suspension culture.
4. Suspension cultures can be multiplied by diluting 5 ml of the original strained suspension culture to 50 ml total volume (1:10 dilution, vol:vol) of fresh medium. The growth rate and cell generation time can be determined.

Usefulness of Suspension Cultures

Suspension cultures are used in a number of biotechnological procedures such as inoculum for plant bioreactors, which resemble biofermentors. Valuable plant products can be extracted from bioreactors during the growth of plant cells as cell suspensions. This type of biotechnology is still in the initial stages of development and industrial scale-up. Suspension cultures treated with enzymes that degrade cell walls produce protoplast cultures which can be used in DNA transformation experiments as well as in protoplast fusion (somatic hybridization) experiments.

Experiment 5: Anther Culture

Background

The purpose of anther and pollen culture is the production of haploid plants by the induction of embryogenesis from repeated divisions of microspores or immature pollen grains (Dodds and Roberts, 1985). The chromosome complement of these haploids can be doubled by colchicine treatment or other techniques to yield fertile homozygous diploids. The resultant diploids can be used in plant breeding to improve crop plants (Sunderland and Cocking, 1978).

The haploid nature of embryoids should be determined by standard chromosome staining procedures (acetocarmine or Feulgen reaction) prior to colchicine treatment. Similarly, the effect of colchicine on chromosome doubling needs to be monitored by chromosome staining.

Materials

tobacco buds
sharp pointed forceps, surgical scissors
95% ethanol
petri dishes of culture medium (1/2 strength MS, 2% sucrose 0.8% agar, glutamine [800 mg/liter], serine [100 mg/liter])

Methods

1. Obtain two buds at the appropriate stage. This occurs in tobacco when the sepals and the petals in the bud are the same length.
2. Holding the bud by the pedicel between the thumb and first finger, dip the entire bud in 95% ethanol for 15 seconds
3. Remove bud and allow excess alcohol to drip off.
4. With a pair of sterile forceps, remove the outer layer of tissue, the sepals.
5. Next, remove the inner layer of tissue, the petals, exposing the anthers.
6. Open the petri dish containing the medium for the induction of haploids. Remove each anther from the bud and drop it onto the medium. Do not damage the anther or include any filament tissue.
7. Repeat for another bud.
8. When finished, seal the plates and place in incubator (25°C).
9. In 2–3 weeks examine for somatic embryo initiation. Embryoid-forming cells are characterized by dense cytoplasmic contents, large starch grains and a relatively large nucleus. Embryoids appear opaque among translucent cells. Embryoids also exhibit high dehydrogenase activity and can be detected by tetrazolium staining (Dodds and Roberts, 1985).

Acknowledgements

Paul Bottino (Botany Department, University of Maryland, College Park) provided the sterilization procedure for Experiment 2 and Methods for Experiments 4 and 5.

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APPENDIX A
Expected Results of Experiment 1

	<i>Control (M)</i> (minerals only)	<i>Organic (O)</i> (minerals, sucrose, vitamins)	<i>B-deficient (-B)</i> (minerals, sucrose)
<i>Shoot</i> (in light)	Callus formation at the cut end; root formation from callus; shoot is totipotent; new leaves, green; plant filled 0.5 of test tube at end of experiment	Callus formation on cut end; root formation; shoot is totipotent; new leaves, green; plant filled <i>whole</i> test tube	Callus formation; root formation; shoot is totipotent; new leaves, green; plant filled 1.0–0.75 of test tube
	Least growth but a complete autotroph	Grew best with sucrose and vitamins that the plant did not need to be synthesized	Less growth than organic but more growth than mineral control
<i>Root</i> (in light and darkness)	1.5X original biomass	10X original biomass	3-5X original biomass
	Minimal growth and no further morphogenesis (no root hairs, no root branches); not totipotent (no green tissue, only white roots)	Growth of original explant and morphogenesis into an entire root system but not totipotent (no green tissue or shoot-like growth)	Growth of original explant; some root hairs; few or no branches; not totipotent
		Roots are heterotrophic and grew best with organics supplied	B-vitamins are essential to root growth and morphogenesis

APPENDIX B
Media Formulae Used in the
Culture of Aseptic Seedling Explants (Root and Shoot Tips)

Mineral Medium = M = white cap
Murashige and Skoog Salt Base (MS)
with Agar

<i>Components</i>	<i>mg/liter</i>
NH ₄ NO ₃	1, 1,900.000
KNO ₃	650.000
CaCl ₂ (Anhydrous)	333.000
MgSO ₄ (Anhydrous)	181.000
KH ₂ PO ₄	170.000
FeHaEDTA	36.700
H ₂ BO ₃	6.200
MnSO ₄ ·H ₂ O	16.900
ZnSO ₄ ·7H ₂ O	8.600
KI	0.830
Na ₂ MoO ₄ ·2H ₂ O	0.250
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025
Sub Total	4,303.530
Agar (non-nutritional gel)	10,000.000
Total	14,303.530

Reference: Murashige, T., and F. Skoog.
1962. *Physiologia Plantarum*, 15:473-497.

Murashige Minimal Organic Medium
with Agar and Sucrose = O = blue cap

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
i-Inositol	100.000
Thiamine HCl	0.400
Sub Total	4,403.930
Agar	10,000.000
Sucrose	30,000.000
Total	44,403.930

Reference: Huang, L. C., and T. Murashige.
1976. *TCA Manual*, 3(1):539-548.

B-deficient = -B = red cap
Demonstration Medium

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
i-Inositol	100.000
Sucrose	30,000.000
Agar	10,000.000
Total	44,403.530

See Appendix C, the general nutrient requirements for plant growth.

Both MS and Minimal Organic (O) media are available as preweighed packets from Carolina Biological Supply Co., Burlington, NC. The mineral medium is Murashige and Skoog Salt Base (MS) to which agar has been added. The organic medium is Murashige Minimal Organic Medium with added agar and sucrose; the salts in this medium are the same as those in MS. The organic medium, then, contains a full inorganic complement plus three organic constituents—sucrose (as a metabolic energy source) and two growth factors, inositol and thiamine. The B₁-deficient medium is the same as the Minimal Organic Medium (O) except thiamine is absent. This B-deficient medium is made by using preweighed MS and adding the other components.

Media preparation: Add preweighed salts to nearly one liter of purified water. Mix until dissolved. Add any other ingredients one at a time. Mix each until dissolved. Add agar and the remainder of the water. Heat until boiling begins. Dispense 10-ml aliquots into 20-mm diameter culture tubes. Add cap and autoclave. The pH of tissue culture media should be between 5.0 and 6.5. (These media formulae are printed with the cooperation of Carolina Biological Supply Co.).

APPENDIX C
Nutrient Requirements of Plants for Autotrophic Growth

<i>Nutrients</i>	<i>Assimilation Form</i>	<i>Relative Proportion Required</i>	<i>Function</i>
Nitrogen	NO ¹	160	Structural component of amino acids, nucleic acids, hormones, coenzymes, proteins, etc.
Phosphorus	PO ³	40	Structural components of nucleic acids, phospholipids, ATP, coenzymes, etc.
Potassium	K ⁺	125	Activator of various enzymes; regulation of stomatal opening and closing
Sulfur	SO ²	75	Structural component of amino acids, vitamins, enzymes, etc.
Magnesium	Mg ⁺⁺	50	Cofactor for many enzyme reactions of carbohydrate metabolism; structural component of chlorophyll
Calcium	Ca ⁺⁺	50	Enzyme activator, required for mitosis, forms Ca pectates of cell wall
Iron	Fe ⁺⁺ /Fe ⁺⁺⁺	2	Structural component of hemes (cytochromes, etc.) and electron acceptors in light reaction
Others and Trace Elements		Traces	Mn, Bo, Cl, Na, I, Cu, and Mo enhance activity of various enzymes
Carbon	CO ₂ or HCO		Gets fixed into carbohydrates
Hydrogen Oxygen	as H ₂ O		Solvates constituents of tissues and is split by photolysis-light reaction, photosynthesis

* Number of pounds necessary to produce 100 bushels of corn.

APPENDIX D
Media Formulae Used in the
Culture of Aseptic Seedling Explants "in vitro"

**Murashige Shoot Multiplication
Medium A
(plus added sucrose)**

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
NaH ₂ PO ₄ ·H ₂ O	170.000
Adenine Sulfate	80.000
2iP	30.000
IAA	0.300
i-Inositol	100.000
Thiamine HCl	0.400
Sucrose	20,000.000

**Murashige Shoot Multiplication
Medium B
(plus added sucrose)**

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
NaH ₂ PO ₄ ·H ₂ O	170.000
Adenine Sulfate	80.000
IAA	2.000
i-Inositol	100.000
Kinetin	2.000
Thiamine HCl	0.400
Sucrose	20,000.000

**Murashige Shoot Multiplication
Medium C
(plus added sucrose)**

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
NaH ₂ PO ₄ ·H ₂ O	85.000
Adenine Sulfate	40.000
i-Inositol	100.000
Kinetin	1.000
NAA	0.100
Thiamine HCl	0.400
Sucrose	20,000.000

**Murashige and Skoog Salt Base (MS)
Medium D = Control, inorganic**

<i>Components</i>	<i>mg/liter</i>
NH ₄ NO ₃	1,650.000
KNO ₃	1,900.000
CaCl ₂ (Anhydrous)	333.000
MgSO ₄ (Anhydrous)	181.000
KH ₂ PO ₄	170.000
FeHaEDTA	36.700
H ₂ BO ₃	6.200
MnSO ₄ ·H ₂ O	16.900
ZnSO ₄ ·7H ₂ O	8.600
KI	0.830
Na ₂ MoO ₄ ·2H ₂ O	0.250
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025
Total	4,303.530

**Murashige Minimal Organic Medium
with NaH₂PO₄·H₂O and Sucrose
Medium E
(= Control, organic and inorganic)**

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
NaH ₂ PO ₄ ·H ₂ O	170.000
i-Inositol	100.000
Thiamine HCl	0.400
Sucrose	20,000.000

All media are available as preweighed packets from Carolina Biological Supply Co., Burlington, NC, and other suppliers. Specific additions to preweighed packets, as indicated above, supplement Media A, B, C, and E. *One percent (1%) agar was used to solidify each medium.*

APPENDIX E
*Formulae for Tobacco Callus Initiation Medium and
 Tobacco Shoot Development Medium*

Tobacco Callus Initiation Medium

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
i-Inositol	100.000
Nicotinic Acid	0.500
Pyridoxine HCl	0.500
Thiamine HCl	0.400
Glycine	2.000
Casein Hydrolysate	1,000.000
IAA	2.000
Kinetin	0.200

Tobacco Shoot Development Medium

<i>Components</i>	<i>mg/liter</i>
MS Salt Base	4,303.530
i-Inositol	100.000
Nicotinic Acid	0.500
Pyridoxine HCl	0.500
Thiamine HCl	0.400
Glycine	2.000
Casein Hydrolysate	1,000.000
IAA	0.030
Kinetin	1.000

Premade media in sterile culture jars are available from Carolina Biological Supply Co., Burlington, NC, and other suppliers.