Chapter 2

Teaching Concepts of Plant Development With Lettuce Seeds and Seedlings

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David Webb is an Assistant Professor of Forest Biology at the Institute of Paper Science and Technology (IPST). When this workshop was presented (1984) he was an Assistant Professor in the Department of Biology at Queen's University, Kingston, Ontario, where he taught plant development, plant tissue culture, and economic botany. He subsequently worked in the private sector at B.C. Research Corporation, and joined the IPST in 1989 where he is involved in graduate education and research. He also held appointments at the University of Puerto Rico and Simmons College. He received his Ph.D. (1978) in Botany from the University of Montana. His major interests have been in plant development. Since laboratory manuals are usually not available for experiments in this area, he has tried to develop laboratory exercises which were effective in dealing with various physiological aspects of plant development, and would give students the experience performing real experiments. In addition, he has tried to develop inexpensive laboratory exercises. He has contributed to earlier ABLE workshops and has also given presentations to the Teaching Section of the Botanical Society of America.

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Introduction

The use of lettuce seeds and seedlings to illustrate a wide range of plant developmental phenomena will be described in this chapter. Traditionally a variety of higher plants have been used to teach plant development. However, in most cases a greenhouse or some other substantial plant growth facility is required. Furthermore, plant growth responses typically take weeks to become evident. These factors, as well as the lack of repeatability of many experiments, serve to restrict the use of plants in teaching developmental concepts. Additionally, I thought that there might be some value in using one plant system for a range of experiments. This should allow students to more readily integrate different biological concepts. I also try to give students laboratory activities which approximate research experiments, and I wanted a system which would be quantitative as well as rapid, easy, and inexpensive. Finally, it would be desirable if the system could be used for student-designed research projects.

My ideas for such a system started to crystallize after I attended a workshop by V. K. Sawhney and T. A. Steeves (University of Saskatchewan) on lettuce hypocotyl elongation at Botany 80 in Vancouver, British Columbia. The exercise in this chapter concerning colchicine is derived from their workshop. Subsequently, I have produced exercises which deal with seed germination, photomorphogenesis, and phototropism, as well as the physiological control of hypocotyl growth and tissue culture differentiation. With the exception of the tissue culture experiments, all of the activities are easy to set up, require little space, and the results can be obtained in a few days. In many cases students can take their cultures home and gather their results independently, and student projects can be readily designed using these exercises as pilot experiments. Even the tissue culture system is comparatively easy to do, and it is extremely reliable and relatively fast. Results can be documented after only 14 days of culture.

The experiments discussed here can be used at all levels of university education, and I have successfully used some of them with advanced high school students. Many experiments are so simple that they can be used to compliment others that you may already be using. Suggestions are provided for further experiments and many more ideas can be derived from the literature cited herein, while others await the creativity of you and your students.

Seed Germination

Seed germination is known to be controlled by a variety of internal and external factors, and some seeds have specific requirements for germination (Bewley and Black, 1978; Mayer and Poljakoff-Mayber, 1982). Many lettuce cultivars, especially Grand Rapids, require light to germinate (Hendricks, 1980). While white light stimulates germination, different spectral regions have conflicting effects. Red light promotes germination, but far-red light reverses the red light stimulation. Germination can be switched on and off by brief exposures to red or far-red light so long as the treatments are given close together. The final outcome depends on the last type of illumination given. Additionally, blue light inhibits germination if given continuously. These experiments are based on the availability of light-sensitive lettuce seeds which are sometimes difficult to locate. Carolina Biological Supply Co. (2700 York Rd., Burlington, NC 27215) frequently carries light-sensitive lettuce seeds, and I have kept seeds for over 5 years with 100% viability by storing them in a refrigerator. Furthermore, seeds of Grand Rapids lettuce which were not initially light requiring became so after several months in a refrigerator. Therefore, if you locate a source of light-sensitive seeds, buy a 1/4 pound or more. Non-light-sensitive seeds will work well for the other experiments described in later sections.

To do light quality experiments one needs inexpensive filters which transmit discrete regions of the spectrum. Edmund Scientific (101 E. Glouchester Pike, Barrington, NJ 08007) sells inexpensive cellulose acetate filters which can be used to isolate the blue, green, red, and far-red regions of the spectrum (Webb, 1981). Fluorescent lights are good sources of blue, green, and red light, but, except for grow-lite wide-spectrum tubes, do not emit far-red irradiation (Klein, 1973). Incandescent bulbs are excellent sources for far-red light. For blue light I use one layer of the Dark Urban Blue (#866) filter; for green I use one layer of the Medium Green (#874) filter; and for red, I use two layers of the Medium Red (#823) filter. Two layers of the red filter are used because it transmits small amounts of blue light. For all the above light qualities I have used cool white fluorescent tubes; warm white and grow-lites should work just as well. For far-red light, I combine the blue filter with two layers of red filter and use a 60-watt incandescent bulb.

Old typewriter paper boxes make ideal light-exposure chambers. For top illumination, cut away most of the top, but leave a 1-cm margin all the way around. Cut your filters so that they completely cover the top of the box; seal inside and outside with black plastic tape. If the box fits tightly no further precautions are necessary to prevent light leakage. For long term experiments you may wish to seal the margin along the bottom of the box with black tape. Also check the corners for possible light leaks. Seal these inside and out with black tape. Construction of each light box takes 15–20 minutes, and they can be used repeatedly. When the boxes wear out, the filters can be recovered and used again.

Effect of Continuous Irradiation

Light-sensitive seeds are stimulated to germinate by placing them on moist filter paper (5 ml of distilled H2O) in a standard petri dish (100 mm × 15 mm) for 1 hour with approximately 250 ft.-c. fluorescent light. Normal classroom light will probably suffice, but two 20- or 40-watt fluorescent tubes positioned 50–100 cm above the petri plates are more than adequate. For white-light intensity measurements I use an inexpensive General Electric foot-candle meter which is available from Carolina Biological Supply Co. If you use disposable petri plates you may need to put the filter paper in the top for a proper fit. I adjust the pH of my solutions to 5.5–6.0. Other pH values may work, but it is important to be consistent. While 1 hour is recommended, you should check

your seed lot to be sure that this will yield near 100% germination upon subsequent dark incubation.

Students transfer 10–20 light-treated seeds to moist filter paper in petri plates. These are exposed for 48 hours to continuous white (fluorescent 250 ft.-c.) light, blue, red, or far-red light, and to darkness. For far-red light, position the incandescent bulb 50 cm above the far-red light box. Since the filters decrease the total amount of light entering the coloured light treatments, I tape a piece of bond paper over the white light box to reduce the white-light intensity. The dark box can be wrapped in foil, and all boxes should be exposed to similar environments. This experiment could be refined by carefully considering the effects of light intensity on germination, but this would require a quantum radiometer. Furthermore, the response is saturated at fairly low energies, and this consideration will not obscure the results.

Root emergence after 48 hours is the criterion used to score germination. Typical class results are given in Table 2.1, and as expected blue and far-red light inhibited germination while white and red light were promotive or had no effect. You may wish to include a dark control which had no light exposure (Table 2.2) if this is the only experiment of this type you are doing.

Table 2.1. Percent germination of lettuce seeds after 48 hours
of continuous light or darkness following 1 hour of white light.

Dark	White light	Red light	Far-Red light	Blue light
100	100	99	0	0

Effects of Brief Red and Far-Red Illumination

In this experiment, one seed transfer must take place in darkness. It is helpful to have a small darkroom available to do this. However, I have done the transfer in class by using two large black trash bags placed one inside the other.

Seeds are incubated in petri plates for 1–2 hours in darkness. One set remains in the dark as a control, and one set is exposed to continuous white fluorescent light (250 ft.-c.). One set is exposed to red light for 5 minutes then placed in darkness. The 5-minute exposure was determined empirically for a particular seed lot, and you would need to test any seed lot to know how much time would be required to yield a maximal response. To expose seeds to red light, place your petri plates plus seeds into the red light box, add 5 ml of distilled H_2O to each plate, cover the plates, put on the box top, and cover with aluminum foil. Place this in a dark place for 1–2 hours. For exposure, simply place the box under your fluorescent lights (250 ft.-c.), remove the foil for 5 minutes, replace the foil and place in darkness. Exposure to far-red light is achieved in the same way as red light exposure, except that you must use a 60-watt (100-watt is okay) incandescent bulb positioned about 50 cm above the far-red light box.

To study the reversibility of red light-stimulated germination by far-red light, first expose the seeds to 5 minutes of red light. Take the box to a dark room (or use large black plastic bags) and replace the red filter top with a far-red filter top, then expose to far-red light for 5 minutes. Finally, cover the box with foil and incubate in darkness for 48 hours.

Typical class results are presented in Table 2.2. This experiment could easily be extended by having a red, far-red, red treatment, and so on. The final outcome should depend on the last light treatment given. You could also ask the question when does far-red light loose its ability to reverse the effects of red light. Simply illuminate seeds with red light and apply far-red light either immediately, or after 10 minutes, 30 minutes, 60 minutes, 2 hours, 4 hours, etc. You could also ask when do the seeds become competent to respond to red light. Allow seeds to hydrate in

darkness for different periods of time before exposing them to a saturating dose of red light. Many other ideas can be obtained by consulting the references in Hendricks (1980).

Table 2.2. Percent germination of lettuce seeds following 1 hour
of dark hydration and exposure to light or darkness.

Dark	Continuous white light	5 minutes of red light	5 minutes of red and 5 minutes of far-red light	5 minutes of far-red light
1.0	100	100	0	0

Effects of Plant Hormones

In many cases the germination requirement for light can be overcome by gibberellic acid (GA3). In other cases, a cytokinin like kinetin (K) can stimulate dark germination. Abscisic acid (ABA) typically inhibits germination but this inhibition can be overcome by other hormones (Bewley and Black, 1978; Mayer and Poljakoff-Mayber, 1982; Poggi-Pellegrin and Bulard, 1976).

Effects of GA3 and K

Seeds are placed on filter paper in petri plates. Five ml of test solution is added to the appropriately labelled plate which is wrapped in foil or placed in a light-proof box and then placed in a dark space for 48 hours. Typical class results are given in Table 2.3 and show the promotive effect of GA. Dark and continuous light controls are included. The effects of individual hormones and hormone combinations can also be examined (Table 2.3). In this experiment although K did not significantly stimulate germination by itself, K stimulated germination when added with a sub-optimal GA concentration (Skinner and Shive, 1958).

Interactions Between ABA, GA, and K (Poggi-Pellegrin and Bulard, 1976)

Seeds are hydrated in petri plates with 250 ft.-c. cool white fluorescent light for 1 hour to stimulate germination. Ten to 15 seeds are transferred to petri plates containing filter paper moistened with 5 ml of test solutions. GA and K are used at $5 \times 10\text{-}5$ M, and ABA is tested over a range of 2–10 µM (1 µM = $1 \times 10\text{-}6$ M). Each hormone is tested individually and in all possible combinations.   Inoculated plates are incubated in darkness for 48 hours and scored for germination. Results from two seed lots (SB = cultivar Salad Bowl; GR = cultivar Grand Rapids) are presented in Figure 2.1. Both seed lots require light to germinate. ABA inhibits germination with both seed lots, but GR is inhibited only by the highest ABA concentration tested, while SB is more sensitive to ABA. K overcomes inhibition by ABA but GA is not very effective. With SB, the K-GA combination is more effective than K alone, but this not clear for GR. Both sets of results are included as a caution to remind you to test the experiment before giving it to the class. The results with SB are easier to interpret and more consistent with those in the literature.

Table 2.3. Percent germination of lettuce seeds in darkness
after 48 hours in gibberellin or kinetin.

Hormone	Molarity				
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Gibberellin	100	100	25	0	0
Kinetin	0	15	10	15	5
Distilled H2O in dark: 10% germination					
Distilled H2O in light: 100% germination					
Kinetin 1×10^{-4} M and gibberellin 5×10^{-6} M: 75% germination					

It would be interesting to test the effects of these hormones on dark germination of light-requiring seeds (Table 2.3) or of light-insensitive seeds. To get effective dark germination for light-requiring seeds the GA3 concentration would need to be raised to 10^{-4} M. Other ideas can be gained from the article by Poggi-Pellegrin and Bulard (1976).

GA and ABA stock solutions can be prepared by dissolving them in 0.1 N NaOH. Kinetin and other cytokinins are soluble in 0.1 N HCl. Apply heat if cytokinins do not dissolve readily. There are many other chemicals which could be tested on lettuce seed germination, and some of the metabolic inhibitors mentioned in the next section would be good candidates.

Test solutions can be made by dilution:

1.   To make 5×10^{-5} M GA dissolve 0.0173 g GA3 in 100 ml. 0.1 N NaOH (= 5×10^{-4} M stock). Use 10 ml of this to make 100 ml of 5×10^{-5} M GA test solution.

2.   For K, dissolve 0.01076 g in 100 ml of 0.1 N HCl (= 5×10^{-4} M stock). Use 10 ml of this to make 100 ml of 5×10^{-5} test solution.

3.   For ABA, dissolve 0.0026 g in 100 ml of 0.1 N NaOH (= 10^{-4} M stock). Use 2 ml/100 ml to make your 2 µM solution. Use 5 ml/100 ml or 10 ml/100 ml, respectively, to make the 5 and 10 µM test solutions.

4.   To make combinations simply calculate the volumes of hormone stock solutions needed/100 ml and subtract this total from 100 ml to find the required volume of distilled H2O. For example to make the GA, K, and ABA 5 µM solution you would need 10 ml of GA stock + 10 ml K stock + 5 ml ABA stock = 25 ml + 75 ml distilled H2O = 100 ml.

5.   Since the hormones are dissolved in acid or base, it is important to adjust the final pH to some uniform value. I typically use 5.7 but other values near neutrality should be okay.
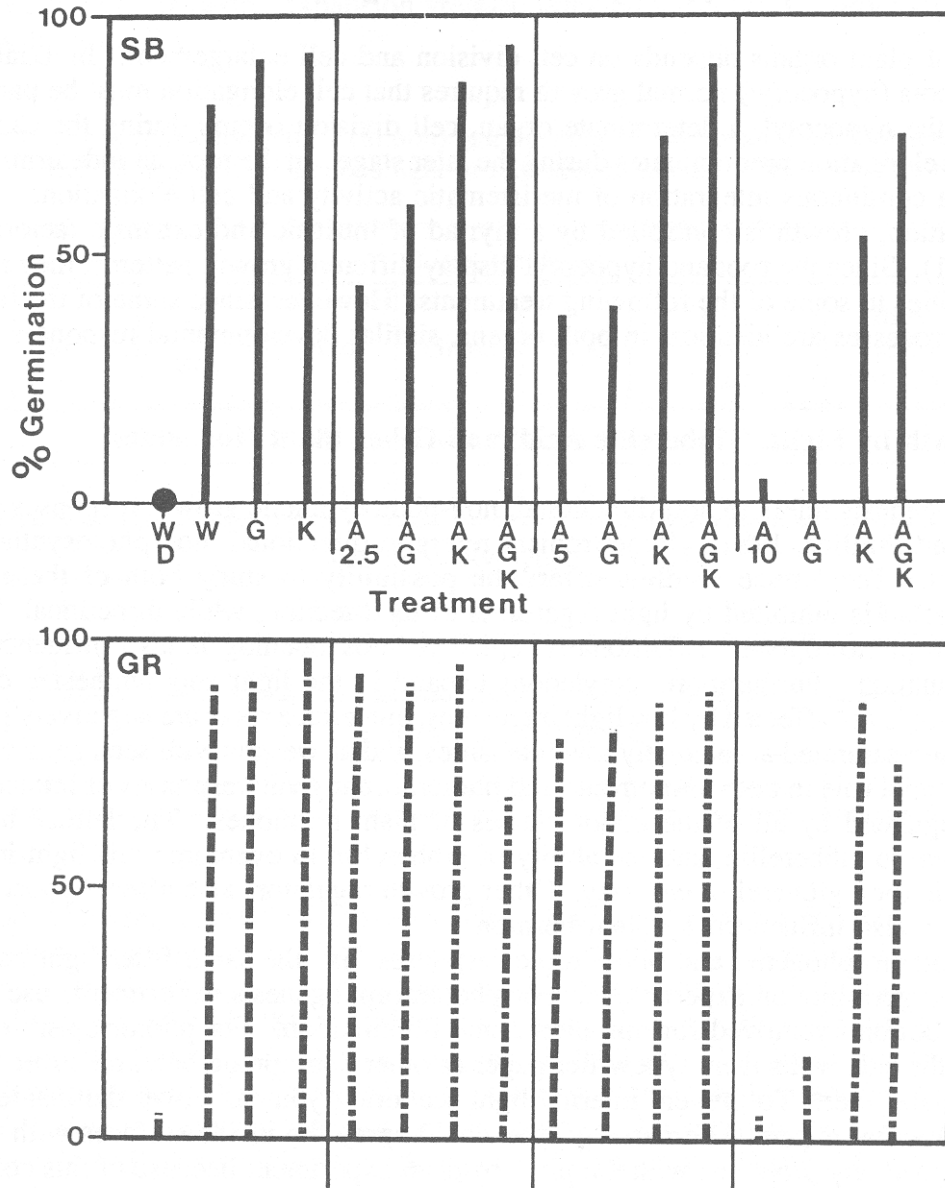
Figure 2.1. Percent germination of Salad Bowl (SB) and Grand Rapids (GR) lettuce seeds after 48 hours in darkness on test solutions. Seeds were hydrated for 2 hours in water with 250 ft.-c. fluorescent light. WD represents the germination of seeds not exposed to light and cultured in distilled water. W = water; G = GA3 at 5 × 10-5 M; K = kinetin at 5 × 10-5 M; A2.5 = ABA at 2.5 μM; A5 = ABA at 5 μM; A10 = ABA at 10 μM. All treatments for a particular ABA concentration are grouped together in boxes. Treatments were identical for both seed lots. The pH of each solution was 5.7.

Hypocotyl and Root Elongation

The growth of plant organs depends on cell division and cell enlargement. In axial structures like roots and shoots (hypocotyl) normal growth requires that cell elongation must be parallel to the growth axis. In the hypocotyl, a determinate organ, cell division occurs during the early stage of growth while cell elongation predominates during the later stage. In the root, an indeterminate organ, growth requires a continuous integration of meristematic activity and cell elongation.

Like germination, growth is controlled by a myriad of intrinsic and extrinsic factors (Wareing and Phillips, 1981). Since the root and hypocotyl display different growth patterns, they also display different sensitivities to some of the following treatments. However, since some of the fundamental cellular growth processes are identical in both organs, similar developmental responses also occur.

Control of Growth by Light, Gibberellic Acid, and Other Plant Hormones

Photomorphogenesis refers to non-directional, non-photosynthetic growth responses of plants to light (Wareing and Phillips, 1981). Phototropism refers to directional, non-photosynthetic growth responses to light. The lettuce seedling offers the possibility to study both of these processes. Hypocotyl elongation is inhibited by light regardless of its direction, while directional illumination results in positive phototropism. Additionally, epicotyl hook opening is also influenced by non-directional illumination. Furthermore, cotyledons expand in the light and synthesize chlorophyll. Overall root growth is not affected by low light intensities, but lettuce roots are negatively phototropic. These responses are saturated at relatively low intensities, and as we saw with seed germination, light quality plays a crucial role in both phototropic and photomorphogenic responses of lettuce seedlings.

Growth is regulated by all of the major classes of plant hormones. The lettuce hypocotyl is extremely sensitive to gibberellin, and the ability of gibberellin to overcome this light-inhibition of growth is the basis for a gibberellin bioassay. Other growth regulators also affect hypocotyl growth, and root growth is also influenced by plant hormones.

To study photomorphogenic and phototropic responses, use the same filter-light combinations described for seed germination experiments. For photomorphogenesis experiments use light boxes with the tops or bottoms removed for non-directional illumination. For phototropism experiments cut away one of the side walls from typewriter paper or other appropriate box and cover the opening with the appropriate filter. To prevent internal light scattering you can cover the inside of the box with black construction paper. Alternatively, you could spray the inside surfaces with black spray paint. I have not had any problems with the phototropism experiment because of this consideration, and have not bothered to blacken the inside of my light boxes.

Sample results for two photomorphogenesis experiments (A and B) are given in Table 2.4. To set up the experiments, seeds were hydrated for 1 hour under 250 ft.-c. fluorescent light and allowed to germinate overnight in darkness. Germinated seeds (10–15/plate) with roots of 1–2 mm are placed on moist (5 ml of distilled H2O) filter paper in petri plates. These are incubated under light conditions similar to those described for the seed germination experiments. Results are tabulated after 48–72 hours.

Hypocotyl elongation is inhibited by white, far-red, and blue light. Red light is not inhibitory and counteracts the blue light inhibition (Table 2.4). In this case the middle of the box had a red filter which was flanked on both sides by blue filters. Gibberellin overcomes the light inhibition (Table 2.4; Figures 2.2 and 2.4). The ability of GA3 to stimulate hypocotyl elongation in the light follows a clear dose response relationship (Figure 2.2). GA also stimulates elongation in the dark

(Table 2.4; Figures 2.3 and 2.4). The amount of dark stimulation increases over time. Opening of the epicotyl hook (Table 2.5) and cotyledon expansion are also affected by light. Chlorophyll synthesis is also regulated by light quality.

Table 2.4. Mean hypocotyl length (in mm) and opening of the epicotyl hook of lettuce seedlings after 48 hours of continuous light or darkness following 1 hour of white light.

Light condition								
Dark	White	Far-Red	Red	Green	Blue	Blue and Red	Blue and GA (10-5 M)	Far-Red and GA (10-5 M)
Mean hypocotyl length (Expt. A)								
8	3.5	4.5	5.5	–	3.5	–	–	–
Mean hypocotyl length (Expt. B)								
9	2.2	3.8	8.6	7.7	3.3	7	11.5	11.9
Dark and GA(10-5 M) = 11.6								
Epicotyl hook (Expt. B)								
Open	Open	Open	Closed	Closed	Open	Intermediate	Open	Open

Anti-gibberellins (e.g., AMO-1618; available from Rainbow Chemical Co., P.O. Box 31, Northridge, CA) inhibit dark hypocotyl elongation (Figure 2.3). Other anti-gibberellins (e.g., Alar [= B nine] and CCC [= Cycocel]) are available from Carolina Biological Supply Co., Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO 63178), and Aldrich Chemical Co. (P.O. Box 355, Milwaukee, WI 53201, or 1411 rue du Fort, Suite 1403, Montreal, Quebec H3H 2N7). The latter two chemicals are more readily available and should be considered for similar experiments (Baldev and Lang, 1965). Inhibition by AMO-1618 depends on concentration and is overcome by simultaneous GA3 application.

Abscisic acid (ABA) also inhibits dark hypocotyl elongation (Figure 2.4) compared to the H_2O control. ABA inhibition is alleviated by GA3, but hypocotyl elongation is still inhibited compared to the GA treatment. Kinetin has little to no effect on hypocotyl elongation, but strongly inhibits root growth.

ABA also inhibits root growth while GA is slightly stimulatory in darkness. Light (250 ft.-c. fluorescent) also stimulates root growth. Inhibition by ABA and K is not counteracted by GA at least at the concentration tested.

ABA is dissolved in 0.1 N NaOH. Seeds are germinated in the dark overnight following 1 hour hydration in distilled H_2O at 250 ft.-c. fluorescent light. Ten to 15 seedlings with 1–2 mm roots are exposed to each experimental solution (5 ml/petri plate) on filter paper for 48–72 hours in darkness.

Hypocotyl length is relatively easy to measure. The hypocotyl is smooth and colorless in light and darkness, while the roots are hairy and off-white. Also, there is a small swelling where the cotyledons join the hypocotyl. Hypocotyl length is measured from this swelling to the point where the root begins. When the epicotyl hook remains closed it is convenient to measure the hypocotyl length as the distance between the start of the root and the top of the hook. Measurement of the

root is obvious. Consult the article by Thomas et al. (1980) for a discussion of light-dependent gibberellin responses by lettuce hypocotyls. Also refer to articles cited in the next sections.
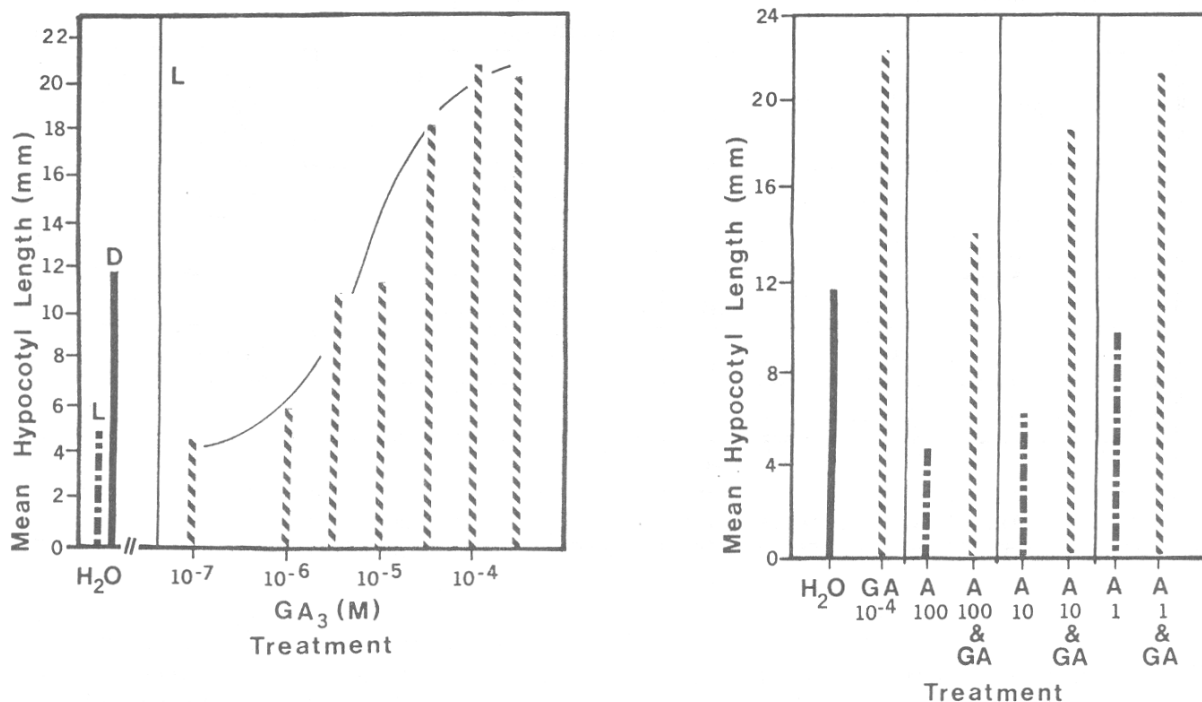

Figure 2.2. (left) Mean hypocotyl length (in mm) for lettuce seedlings grown in water with light (L) or darkness (D) in GA3 solutions in light. Light intensity was approximately 250 ft.-c. Seeds were hydrated for 2 hours with 250 ft.-c. fluorescent light and germinated overnight in darkness. Seedlings with 1–2 mm roots were placed in test solutions for an additional 72 hours.

Figure 2.3. (right) Mean hypocotyl length (in mm) for lettuce seedlings grown in darkness. A100 = AMO-1618 at 100 mg/liter; A10 = AMO-1618 at 10 mg/liter; A1 = AMO-1618 at 1 mg/liter; GA = GA3 at 10-4 M. Seeds were hydrated for 2 hours with 250 ft.-c. fluorescent light and allowed to germinated overnight. Germinated seeds with 1–2 mm roots were used to inoculate cultures which were incubated for an additional 72 hours in darkness.
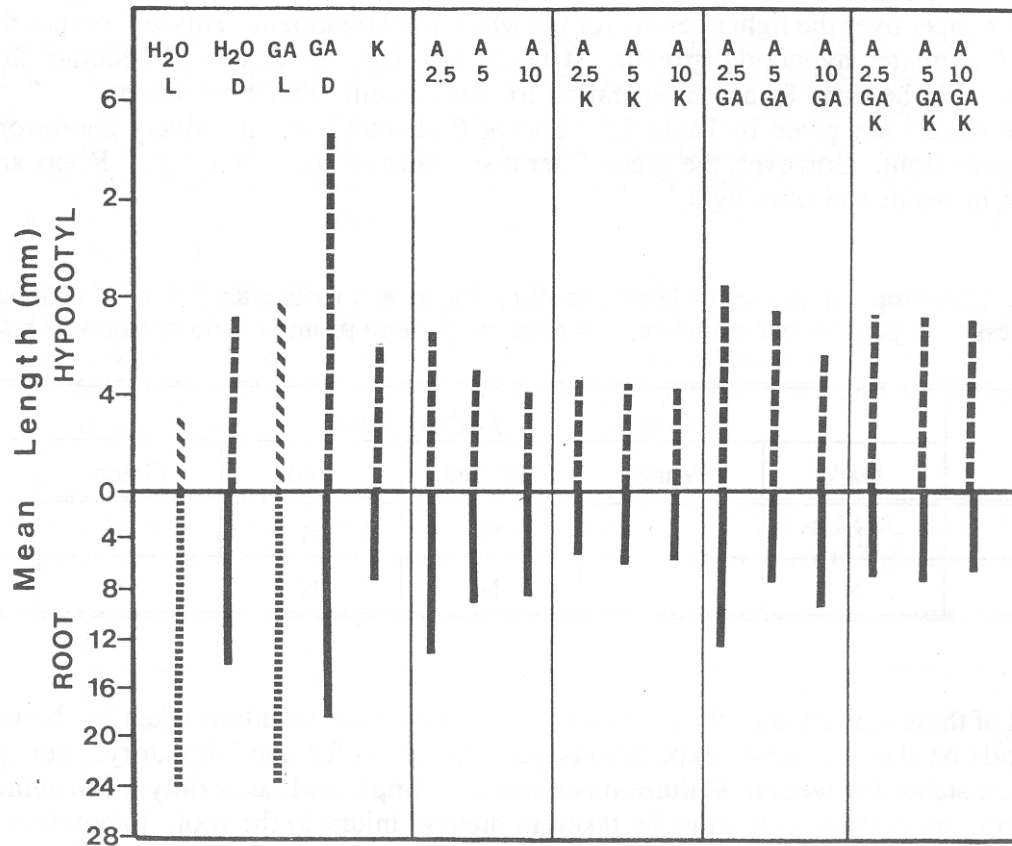
Figure 2.4. Mean hypocotyl (above the abscissa) and root (below the abscissa) lengths (in mm) for lettuce seedlings grown in darkness (D) or with 250 ft.-c. fluorescent light (L). All treatments to the right of the first internal vertical line were grown in darkness. Seeds were hydrated for 2 hours with 250 ft.-c. fluorescent light and germinated overnight in darkness. Germinated seeds were cultured in experimental solutions for 48 hours. GA = GA3 at $5 \times 10\text{-}5$ M; K = kinetin at $5 \times 10\text{-}5$ M; A = ABA, the numbers refer to the concentration (in µM) of ABA.

Hypocotyl and Root Phototropism

To perform the phototropism experiment, seedlings are prepared overnight as just described in the previous section. Ten seedlings are positioned along one line drawn through the center of a petri plate containing 25 ml of 1% plain agar-agar (pH 5.7). Difco Bacto-agar or another semi-purified agar will do. The idea is to hold the seedlings in place so that any directional growth response can be evaluated. It is helpful to draw a line across the outside of the petri dish to act as a guide for planting. After carefully planting the seeds with forceps in the soft agar, tape on the petri plate lid and tape the plate to the bottom of the box so that the seeds are aligned parallel to the open slot on the end of the box. The seedlings will therefore be aligned perpendicular to the direction of the light path. In actuality the lid of the petri plate need not be used so long as the bottom of the plate with the seeds is fastened to the bottom of the light box. However, the lid helps to prevent desiccation. Light boxes are positioned 10 cm away from the appropriate light sources. Fluorescent light is used for white, blue, green, and red light, while incandescent light is

used for far-red light. Place a piece of typewriter paper over the light opening for the white-light treatment. This will reduce the irradiance and allow for greater hypocotyl growth. It takes 4–5 days for results to become obvious. This experiment could be done as a demonstration to compliment other experiments.

Typical results are given in Table 2.5. Shoots (hypocotyl) are positively phototropic in white, blue, and green light. However, the green filter does transmit some blue light. Roots are negatively phototropic in white and blue light.

Table 2.5. Phototropic responses of lettuce seedling shoots and roots after 5 days of continuous light or darkness (+ = positive phototropism; - = negative phototropism; N = no phototropic response).

	Light condition					
	Dark	White	Far-Red	Red	Green	Blue
Shoots	N	+	N	N	+	+
Roots	N	-	N	N	N	-

For all of these experiments the preparation of experimental solutions takes 2–4 hours. The actual time depends on the number of experiments you plan to do for one laboratory. Refrigerated stock solutions are stable for weeks. Culture inoculation is simple and takes only a few minutes per petri plate. During inoculation care must be taken to prevent injury to the root. Laboratory organization could be a problem if too many experiments are attempted during one period. However, different teams of students can set up different experiments. It is a good idea to have at least three separate repetitions of each experiment per lab section to avoid confusion if one group fails to conduct the experiment properly, or makes inaccurate measurements. Although the measurements are simple, sufficient time must be allowed to achieve accuracy. Hypocotyls and roots can be curved and need to be strengthened against a ruler with forceps and a dissecting needle. Forceps should also be used to inoculate cultures. Seedlings can be carefully dropped onto the test solutions so that forceps do not become contaminated with different chemicals. Allow 15–20 minutes for a student to measure the roots and hypocotyls for one treatment. It is also possible to have students make their measurements outside of regular laboratory time. However, this clearly depends on the attitude prevalent among the students in your department. Personally, I try to avoid this type of situation. One alternative is to set up some of the cultures yourself and simply have the students record the results. However, I have found that it is best to have the students set up as many of the experiments as possible. These experiments are ideal if you have two 2-hour laboratories per week. Finally, one lecture period could be used to set up the experiments for analysis later in the week.

Effects of Colchicine and 2-Thiouracil on Hypocotyl and Root Growth

All developmental processes are controlled at many biochemical levels. The use of metabolic inhibitors allows for the exploration of developmental control. Colchicine inhibits microtubule assembly and thus interferes with cell division and directed cell enlargement. In the presence of colchicine, growing cells tend to become isodiametric rather than elongated in any one direction. Since microtubules compose the mitotic spindle, meristematic activity is arrested in the presence of colchicine. Halogenated derivatives of uracil are known to inhibit effective RNA or DNA synthesis. RNA synthesis is inhibited by 2-thiouracil.

Results from two class experiments (A and B) with colchicine (10-3 M) and GA3 (10-5 M) are presented in Table 2.6. Colchicine inhibited hypocotyl elongation and increased hypocotyl width in both light and darkness. However, GA caused a small growth stimulation in the light when given with colchicine. Root elongation was also severely inhibited by colchicine and the subapical region was markedly swollen. Results for root growth are not presented.

Table 2.6. Results from two class experiments with colchicine (10-3 M) and GA3 (10-5 M): mean hypocotyl length and width after 48 hours for lettuce seedlings exposed to light for 1 hour.

Treatment	Light				Dark			
	H2O	GA 10-5	Col 10-3	GA and Col	H2O	GA 10-5	Col 10-3	GA and Col
Length (mm)								
Expt. A	5.4	16.8	2.6	4.1	15.7	16.2	4.3	4.3
Expt. B	9.7	16.0	2.8	3.4	13.0	14.0	3.2	3.5
Width (mm)								
Expt. A	0.92	0.59	1.0	1.8	0.87	0.91	1.5	1.8
Expt. B	0.9	0.8	1.3	1.8	0.9	1.0	2.2	2.1

The effects of colchicine and GA treatment on cell shape are readily visualized. Simply cut out a 5-mm piece from the middle of the hypocotyl and mount it sideways in a drop of water on a microscope slide. Add a coverslip and gently flatten the section so that the cells can be viewed longitudinally. Since the hypocotyl is translucent, it is possible to see the cell outline of the epidermis. These cells are isodiametric, or nearly so in the colchicine treatments and greatly elongated in the GA treatments without colchicine and to a lesser extent in the dark control. It is possible to focus through the epidermis and observe the cortical cells which are larger than the epidermal cells. It helps if you close down the substage iris diaphragm so that the cell walls are more distinct. I have tried a number of common stains, but none have given a significant improvement over unstained material. It has been suggested that safranine is a good stain to try.

The specificity of this response can be checked by testing other microtubule inhibitors, such as vinblastine or the colchicine analog, lumi-colchicine (Keith and Srivastava, 1978). The effects of colchicine can be compared with the microfilament inhibitor cytochalasin B (Sawhney and Srivastava, 1974a; Sawhney et al., 1977). For general references on the effects of GA and colchicine with lettuce seedlings consult Kordan (1980) and Sawhney and Srivastava (1974b, 1975).

Root elongation is markedly inhibited by 2-thiouracil (2-tu) and the root is far more sensitive than the hypocotyl (Tables 2.7 and 2.8, and Figure 2.5). However, hypocotyl elongation is inhibited at high 2-tu concentrations ranging from 2.5×10^{-4} M to 5×10^{-4} M. Uracil slightly stimulates both root and hypocotyl growth and largely overcomes the inhibitory effects of 2-tu. The inhibitory effects of 2-tu on the hypocotyl are more obvious with prolonged culture, but root growth inhibition is evident after only 48 hours (Tables 2.7 and 2.8, and Figure 2.5). Although growth is inhibited, the seedlings appear normal otherwise. It would be interesting to test other uracil analogs, including 5-fluorodeoxyuridine, an inhibitor of DNA synthesis whose inhibition of hypocotyl elongation is only counteracted by thymidine (Katsu and Kamisaka, 1981). Experiments on the kinetic effects of cycloheximide, puromycine, and 5-fluorouracil on lettuce hypocotyl elongation are described by Sawhney et al. (1977). The effects of 2-tu and uracil on root growth are described by Woodstock and Brown (1963). These substances also affect lettuce seed germination and are discussed by Kahn (1966).

Colchicine and uracil are very soluble in H2O, while 2-tu is not very soluble but can be dissolved to make a 10^{-3} M stock solution. Colchicine is a carcinogen and should be handled with care.

Table 2.7. Hypocotyl length (in mm) of lettuce seedlings
after 96 hours in darkness following 1 hour of white light.

H2O	Uracil (10-3 M)	2-thiouracil (10-4 M)	2-thiouracil (2 × 10-4 M)	2-thiouracil (10-4 M) and uracil (10-3 M)	2-thiouracil (2 × 10-4 M) and uracil (10-3 M)
13.9	13.5	11.6	8.3	14.9	14.0

Table 2.8. Root length of lettuce seedlings after 96 hours in darkness
following 1 hour of white light.

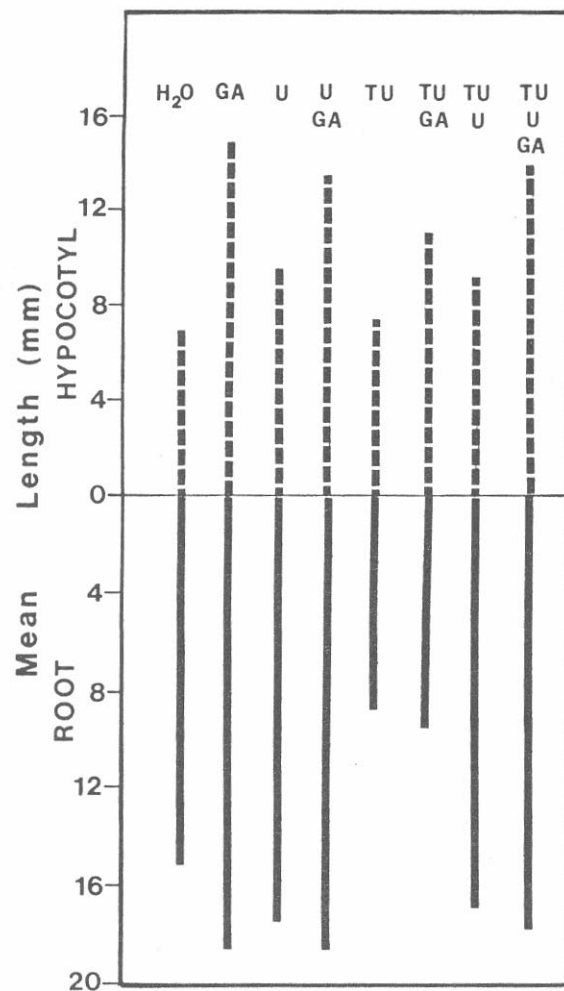| H2O	Uracil (10-3 M)	2-thiouracil (10-4 M)	2-thiouracil (2 × 10-4 M)	2-thiouracil (10-4 M) and uracil (10-3 M)	2-thiouracil (2 × 10-4 M) and uracil (10-3 M)
52.5	46.2	14.7	5.1	37.0	36.0

Figure 2.5. Mean hypocotyl and root lengths (in mm) for lettuce seedlings grown in darkness. GA = GA3 at 5 × 10-5 M; U = uracil at 10-3 M; TU = 2-thiouracil at 5 × 10-4 M. Seeds were hydrated for 2 hours with 250 ft.-c. fluorescent light and germinated overnight in the dark. Seedlings with 1–2 mm roots were placed in test solutions and incubated for 48 hours in darkness.

Tissue Culture

    Lettuce seedlings provide an excellent system for studying organ initiation. The various factors controlling lettuce organogenesis are discussed by Doerschug and Miller (1967) and Webb et al. (1984). Organ-forming potential is influenced by a variety of factors including genotype, age and physiological status of the donor plant, culture medium, culture environment including light, temperature, and atmosphere, as well as the phytohormones used (Murashige, 1974). Lettuce seedlings can be used to explore all of these problems and more.
    Use Grand Rapids lettuce for these experiments. Other cultivars may not work as well. Seeds are disinfected by rinsing in 95% ethanol for 5 minutes followed by soaking in 10% commercial bleach for 20–30 minutes. Prolonged bleach treatment will kill the seeds. Disinfection should be done in a small screw-cap vial, and the vial should be completely filled with bleach. It is the escaping chlorine gas which disinfects the seeds. After bleach treatment the seeds should be rinsed 3–5 times with sterile H2O. Use a sterile spatula to prevent excessive seed loss during

rinsing or wrap the seeds in a small bag of cheesecloth during this entire process. Water can be sterilized by autoclaving or by filtration with a 0.2 μ membrane filter. Instruments are sterilized by keeping them in 95% ethanol. Excess alcohol is removed by passing the instruments through a flame. Do not hold the instruments in the flame so that overheating occurs. Hot instruments will kill the plant tissue. This is more of a problem in later operations.

After rinsing, seeds are transferred to bottles or vials with 15 ml of 1% agar-agar (pH 5.7). I routinely use Difco Bacto-agar. Seeds are incubated with 12-hour photoperiods of 100–250 ft.-c. fluorescent light. Four- to 5-day-old seedlings are the most responsive for shoot formation, and the effects of explant age (Webb et al., 1984) and explant type, root, hypocotyl, or cotyledon (Doerschug and Miller, 1967) make interesting experiments.

Seedlings are removed by grasping their hypocotyls with sterile forceps. Do not grasp the cotyledons. Place them on a sterile surface. We routinely wipe the lab bench top with 95% ethanol to provide a clean working area. Reduced air movement also helps to avoid contamination. We use paper towels autoclaved in foil packets for 40 minutes as sterile working areas. Pre-sterilized petri plates are also satisfactory, but disposable plates are far more expensive than paper towels, and you should use a fresh sterile surface for each set of operations.

Cotyledons are the most responsive seedling organs. These are removed surgically with a sterile scalpel. Care must be taken to not include any part of the hypocotyl with your cotyledon explants. Axillary buds are present at the base of the cotyledons, and these plus the shoot apical meristem will confuse your results if they are inadvertently included. The severed cotyledons should come free from each other if they are properly excised. These are placed with the abaxial (lower or outer surfaces) in contact with the medium. Do not submerge the cotyledons in the medium. As they grow, the cotyledons curve. If the abaxial side is placed against the medium, the cut edge will remain in contact with the substrate, and maximal organ formation will occur. Organogenesis occurs preferentially at the cut edge. Otherwise organogenesis may be severely reduced and retarded. This may not be a major problem since on average half of the cotyledons will probably end up with the preferred orientation, and it is hard to tell the difference between the two surfaces. I usually use a semi-micro spatula to pick up the cotyledons. After flaming, I touch the spatula tip to the surface of the medium in the culture vial (not the seed germination medium). This cools the spatula tip and the adhering moisture (or small piece of medium) helps to pick up the cotyledons. Two cotyledons are transferred to 25 mm × 95 mm shell vials capped with Bellco Kaputs or Magenta 2-way caps (both are available from Carolina Biological Co. but can be purchased less expensively from the manufacturers: Bellco Glass Inc., P.O. Box B, 340A Edrudo Rd., Vineland, NJ, or Gibco Canada Inc., 2260A Industrial St., Burlington, Ont. L7P 1A1, and Magenta Corp., 3800 N. Milwaukee Ave., Chicago, IL 60641). We regularly use Magenta 7-way trays which hold 36 vials or test tubes and are autoclavable. Shell vials are available from most scientific supply companies. Test tubes or screw cap vials can also be used. Erlenmeyer flasks and petri plates also work, but results may vary depending on the type and size of vessel used. However, there will be small variations. Cultures can be sealed with parafilm to prevent evaporation as well as air-borne or insect-borne contamination. Cultures are incubated at 25–27°C with 12-hour photoperiods of 50–250 ft.-c.

The magnitude of shoot formation is regulated by light intensity, photoperiod, and light quality (Kadkde and Seibert, 1977). I use 12-hour photoperiods of cool white fluorescent light of 150–200 ft.-c. Light effects on root formation have not been studied with this system. Light regulation of organogenesis could provide nice experiments for students. A simple foot-candle meter (Carolina Biological Supply Co.) will suffice for measuring white-light levels. The light filters described previously could be used to design simple light-quality experiments.

For media we have used Miller's formulation or Murashige's Minimal Organic Medium (Table 2.9); either works well. The latter is commercially available from Flow Laboratories, 7655 Old Springhouse Rd., McLean, VA 22102, or Gibco Laboratories, 3175 Staley Rd., Grand Island, NY

14072 or 2260A Industrial St., Burlington, Ont. L7P 1A1. I have had success with both but prefer the Gibco product. Agar, preferably purified, is added at 0.8% w/v. Bacto-agar is okay, and other "plant agars" may work. Also, filter-paper supports with liquid medium will work (and the cotyledons float for some time on liquid) and this type of stationary culture could be tried. We use Miller's medium for mineral deficiency studies. We make 100X stocks for each major nutrient and a 100X stock for the micronutrients excluding the iron which is made up separately at 100X. To make FeEDTA stock, dissolve 0.1 g $FeSO_4 \cdot 7H_2O$ in 50 ml of hot water. Also dissolve 0.1338 g $Na_2EDTA \cdot 2H_2O$ in 50 ml of hot water. Combine the two slowly with stirring. A pale yellow solution should result. Wrap this in foil and store refrigerated in darkness. To make a 100X iron stock for Murashige's medium, multiply the amounts of the above two chemicals by 2.78 (Singh and Krikorian, 1980). The vitamins, excluding inositol, are also made at 100X the final medium concentration. These are stored frozen until needed. Inositol is either added as a powder during medium preparation, or a 100X stock solution is freshly prepared. Stocks of the major and minor elements store well in the refrigerator.

For organogenesis, the auxin, indoleacetic acid (IAA), and the cytokinin, kinetin (K), have typically been used with lettuce. These are dissolved in either 0.1 N NaOH or 0.1 N HCl, respectively. Stock solutions of 1.0 mg/liter IAA and 0.1 mg/liter K are convenient. These are added in appropriate amounts after the minerals and vitamins. Sucrose is added at 3% w/v and dissolved. Medium pH is set around pH 6.0 before adding the agar. There is a risk of hydrolysis if the agar is added to an acidic solution. The agar is dissolved with thorough stirring on a hot plate. If the agar burns on the bottom of the flask the medium will be useless. After the agar dissolves, set the final pH to 5.7. Fifteen ml of medium is dispensed to vials, plates, or tubes and autoclaved for 15–20 minutes at 121°C. A longer time may be required for larger volumes. Autoclaved medium is fairly stable but media with IAA are best used promptly or stored in the refrigerator since IAA is photo-labile.

To prepare culture media, calculate the volume of each 100X stock solution and of the hormone stock solutions needed for the total volume of medium. Subtract this sum from the total volume to get the required volume of distilled H_2O. You will need 10 ml/liter of each 100X stock. The volume of hormones will depend on their concentration in the final medium, and this depends on the experiment. Add the dry components and stock solutions to the distilled water one at a time. Remember to add the agar last after the pH has been set to 6.0. Dissolve the agar, set the pH to 5.7, dispense, and autoclave.


Hormonal Control of Organ Formation

With lettuce cotyledons, indoleacetic acid (IAA) stimulates root formation (Figures 2.6 and 2.7), kinetin (K) stimulates shoot formation (Figures 2.8 and 2.9), and the two interact quantitatively to regulate the kind of organ formed. About 50% of the cotyledons form only callus when exposed to K alone (Figure 2.10). The optimal concentration for K is 0.5 mg/liter (2.3×10^{-6} M). The optimal IAA concentration is 5.0 mg/liter (2.85×10^{-5} M). At the optimal K concentration IAA greatly stimulates shoot production. K inhibits root production above 10^{-7} M. At 5×10^{-7} M (0.1 mg/liter) K plus IAA (2.85×10^{-5} M) approximately equal numbers of roots and shoots are formed. Complete plantlet development can be illustrated with regenerated shoots. These root when they are subcultured to medium containing 1 mg/liter IAA. I haven't tried to do this, but leaves from regenerated shoots would probably form shoots on medium with IAA and K. This process could theoretically be continued indefinitely and would illustrate the concept of clonal propagation.

Table 2.9. Components of Miller's Medium (Doerschug and Miller, 1967)
and of Murashige's Minimal Organic Medium.

Component		Concentration (mg/liter)	
		Miller's	Murashige's
Macro-nutrients	H_2PO_4	300	170
	KNO_3	1000	1900
	NH_4NO_3	1000	1650
	$Ca(NO_3)_2 \cdot 4H_2O$	500	–
	$MgSO_4 \cdot 7H_2O$	71.5	–
	KCl	65	–
	$CaCl_2$	–	1900
	$MgSO_4$	–	181
Micro-nutrients	$MnSO_4 \cdot H_2O$	4.9	16.9
	$ZnSO_4 \cdot 7H_2O$	2.7	8.6
	H_3BO_3	1.6	6.2
	NaFeEDTA	13.2	–
	KI	0.75	0.83
	FeEDTA	–	36.7
	$Na_2MoO_4 \cdot 2H_2O$	–	2.5
	$CuSO_4 \cdot 5H_2O$	–	0.025
	$CoCl_2 \cdot 6H_2O$	–	0.025
Vitamins	Myo-inositol	100	100
	Thiamine·HCl	0.2	0.40
	Nicotinic acid	0.5	–
	Pyridoxine·HCl	0.2	–
Carbo-hydrates	Sucrose	30000	30000
	Agar	8000	8000

Other cytokinins, such as benzyladenine (BA), 2-isopentyl adenine (2iP), and zeatin (Z), are either more potent (BA), or less potent (Z and ZiP) than K at inducing shoot formation in the absence of IAA. BA is about 10-fold more potent than K at inducing shoot formation. Determination of optimal cytokinin concentrations for shoot formation would be a worthwhile experiment for students. The interaction of IAA with cytokinins other than K would also be interesting experiments. It would also be of interest to test different lettuce cultivars with K and other cytokinins at their optimal concentrations. Our work suggests that different cultivars do not respond in the same way to cytokinins. Some cultivars form lots of shoots (Grand Rapids), while others form lots of callus (White Boston). The responses of different cultivars to IAA or IAA-K combinations are not known. It would also be possible to test the regenerative capacity of lettuce from the supermarket with cotyledons of the same cultivar. Different auxins like indolebutyric acid, naphthalene acetic acid, and 2,4-dichlorophenoxy acetic acid have different morphogenic potentials. It would be worthwhile to test these auxins at equimolar concentrations alone and with K to compare with their abilities to promote root, or shoot, or callus formation. While a small amount of callus formation occurs at the cut edge of the cotyledon, callus formation is not extensive when IAA and/or K are used. I have found that when petri plates are used, shoot

formation does not occur with K alone, but only callus formation results (Figure 2.10). Otherwise, the results are similar to those in vials.
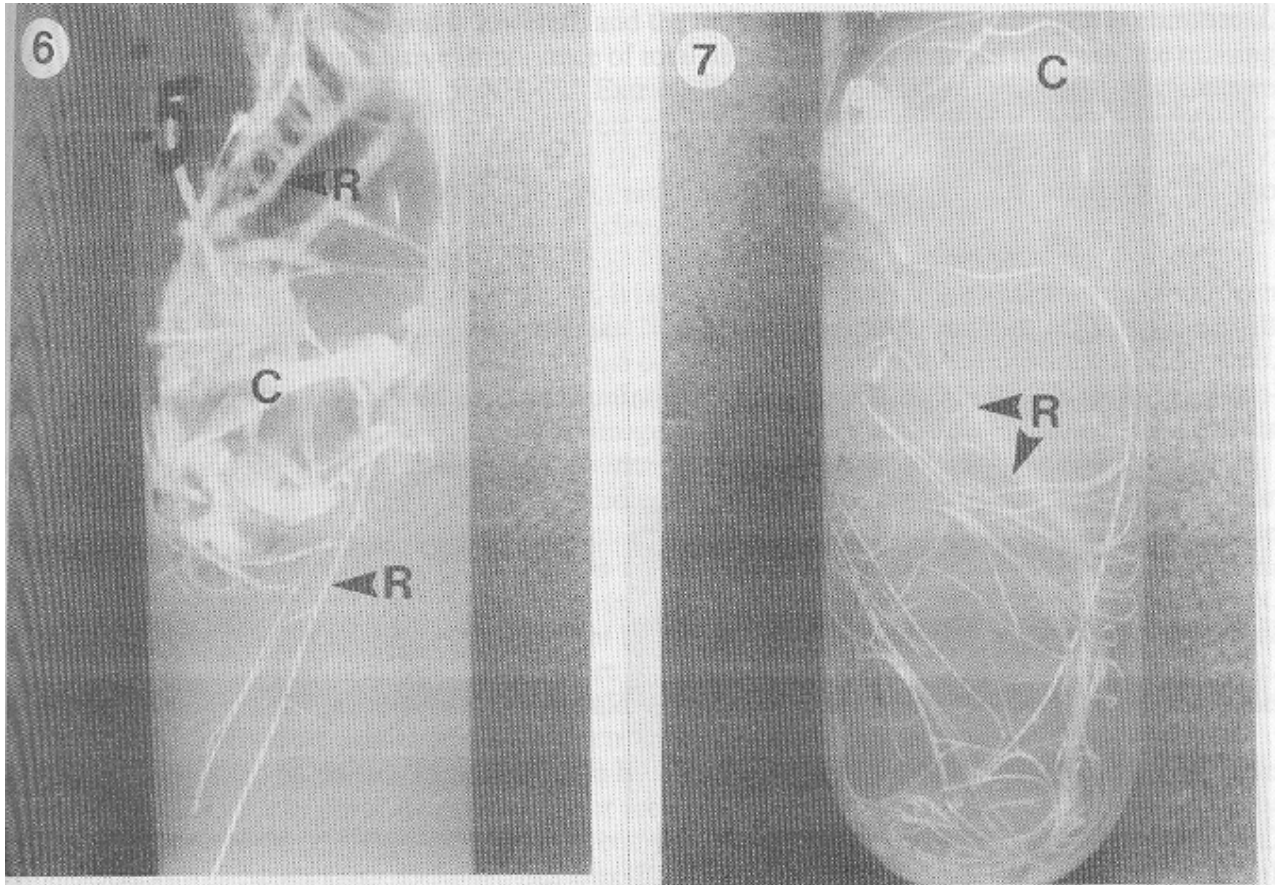
Figures 2.6 and 2.7. Front (Fig. 2.6) and rear (Fig. 2.7) views of root (R) formation by lettuce cotyledons (C) on Murashige and Skong (MS) Medium containing 5.0 mg/liter IAA after 48 days. Note the abundance of root hairs on aerial roots in Figure 6. (Both figures are magnified 1.7 times.)

On IAA, root formation is evident after 7 days. The root hairs are very long and you may mistake them for fungal hyphae. On K and K plus IAA, shoot production is visible after 10–14 days. However, shoots are very small at this time. Good quantitative results can be obtained after 21 days by using a dissecting microscope to count shoots and roots. Large shoots will develop with continued culture, but counting becomes impossible (Figure 2.9). On the growth regulator-free Miller's Medium a few cotyledons produce roots near the mid-vein.

A basic experiment would be to have students inoculate cultures of growth regulator-free Miller's Medium, and media with IAA alone (2.85 × 10-5 M), K alone (2.3 × 10-6 M), IAA plus K at the above concentrations together, and IAA (2.85 × 10-5 M) plus K at 5 × 10-7 M. These treatments should result in a few roots; many roots; a few shoots; many shoots; and many roots plus many shoots, respectively. If counting organs presents a problem, the amount of organ formation can be estimated by ranking. A reasonable system would be 0 = 0, 1 = 1–10, 2 = 11–25, 3 = 26–50, and 4 = 51–100 organs. An average rank can be computed for each treatment. Also the percentage of explants forming organs is a useful and easily obtained value. However, percentage alone can be misleading
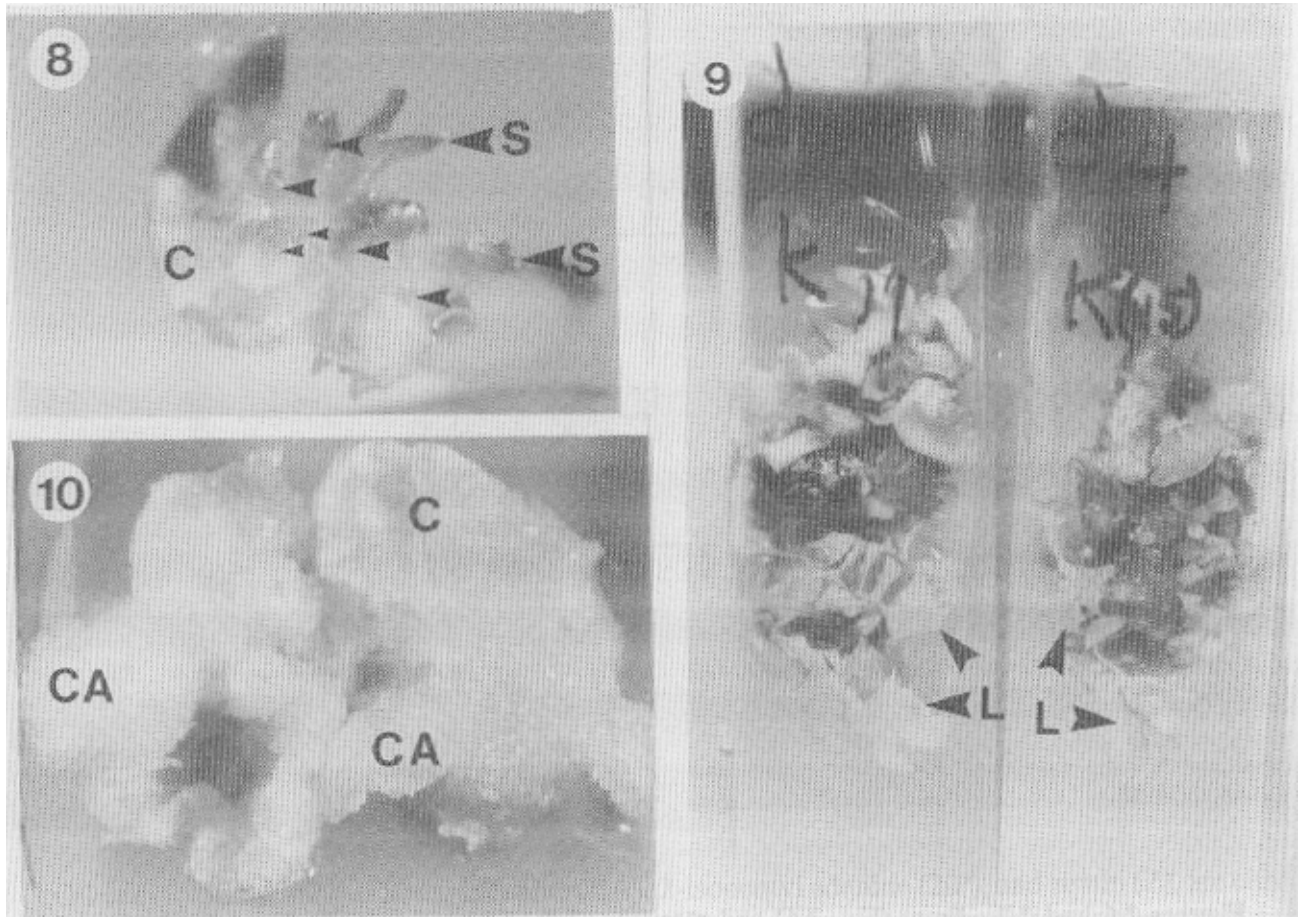
Figures 2.8 to 2.10. Cotyledons (C) cultured on MS Medium containing 0.5 mg/liter K for 21 days (Figs. 2.8 and 2.10) or 48 days (Fig. 2.9). Note the different sizes of shoots (S) present after 21 days in Figure 2.8. Shoots are indicated by small and large arrows. Approximately half of the cotyledons produce shoots and the other half produce callus (CA) as shown in Figure 2.10. After 48 days, a great number of shoots have been produced, and some have developed prominent leaves (L). (Figs. 2.8 and 2.10 are magnified 4.4 times; Fig. 2.9, 1.3 times.)

Mineral Deficiency Experiments

Doerschug and Miller (1967) studied the effects of omitting KH2·PO4, NH4NO3, or FeEDTA from the culture medium. Deletion of any one had severe effects on shoot initiation with K plus IAA. I have repeated these experiments with K alone and with IAA alone. In all cases organogenesis was inhibited by the deletion of any one medium component. Root formation was less sensitive in general, especially to the absence of NH4NO3. The response with K (2.3×10^{-6} M) plus IAA (2.85×10^{-5} M) was similar to that with K alone.

Reduced nitrogen is important for shoot formation in other tissue culture systems. By removing NH4NO3 the total medium nitrogen is lowered, and the only source of reduced nitrogen is eliminated. To discriminate between the relative importance of total nitrogen versus reduced nitrogen, the missing nitrogen can be restored as KNO3 or NH4Cl. Extra KNO3 can be added at 2.5×10^{-2} M to restore the total nitrogen to the level in Miller's complete medium. This does not restore the shoot-forming capacity of the medium, but addition of 2.5×10^{-2} M NH4Cl does restore shoot forming capacity. Other reduced nitrogen sources like (NH4)2SO4, ammonium citrate, and the amide, glutamine, also restore shoot forming capacity to medium deficient in NH4NO3. Consult the article by Gleddie et al. (1983) for other ideas relating to this topic.

With multivalent compounds like (NH4)2SO4 it is important to consider the total amount of nitrogen present as opposed to a univalent compound like NH4Cl. An equimolar amount of (NH4)2SO4 will have twice as much nitrogen as NH4Cl. This same consideration applies to amides such as glutamine.

It would be interesting to determine which amino acids could substitute for NH4 in restoring shoot-forming capacity. Other sources of reduced nitrogen could also be tested. Additionally the effects of other major and minor elements, as well as vitamins, could be explored.

Alteration of medium pH could also be studied with regard to organogenesis. However, agar hydrolyses at acid pH when heated and this would place a lower limit to any pH experiment where agar was used. Since the cotyledons float on liquid medium, this could be used for short term experiments, or filter paper supports could be used with liquid medium for pH or other experiments. Medium pH changes during culture and it might be interesting to see how different treatments affect these changes. With soft agar you can measure the medium pH with a combination pH electrode. Alternatively, the agar from individual tubes could be collected and melted into a large enough volume to measure the pH with a standard pair of electrodes.

Lettuce seeds can be obtained from Carolina Biological Supply Co. (be sure to have them indicate the cultivar at the time of purchase) and all major seed houses. Grand Rapids lettuce works extremely well and is recommended. Two reliable seed companies are: Park Seed Co., Inc., Greenwood, SC 29647, and Stokes Seeds Ltd., 39 James St., Box 10, St. Catherines, Ont. L2R 6R6. We use 125-ml Boston round bottles to germinate the seeds, but petri plates work reasonably well. Medium pH is adjusted to 5.7 with dilute NaOH and HCl. We use clear polypropylene caps because coloured caps are opaque and can cause variation in the amount of light reaching the explants (cotyledons). This won't be a problem if you use petri plates or erlenmeyer flasks. With erlenmeyer flasks you can use cotton, cotton wrapped in cheesecloth, foam, or aluminum foil to cover the opening. Parafilm can also be used, however it will not survive autoclaving. It must be sterilized in 20% commercial bleach and dried in sterile towelling before application if it is used as the only covering. However, it can be applied "as is" to seal the edges of other covers. Polypropylene film (Norprop Films, 601 E. Lake St., Streamwood, IL 60103) which is autoclavable can also be used. This is permeable to 02 and CO2, and 1 mil thickness is recommended (Mahlberg et al., 1980). This film must be secured with a rubber band. Counting can be done through the walls of the container, but for accurate counts the cotyledons must be

removed and teased apart since they tend to curl up, and organs may be formed on all surfaces of the cotyledon. Most of the initial action takes place at the cut edge, but later it spreads to other parts of the cotyledon. The cleft at the tip of the cotyledon is also a very responsive area.

One small experiment could be to determine how small can a piece of cotyledon be cut before it loses its organogenic capacity. One could also ask if there is a difference in morphogenetic capacity between the lower half and the upper half of the cotyledon. Shoots will be at different stages of development (Figure 2.8), and I do not make any distinction between large and small shoots. Also there will be principal roots and lateral roots. I usually count total root number, but you may wish to distinguish between these, or count only principal roots.

We have not had major problems with sterility. I use a regular laboratory with no special precautions, other than those mentioned previously. Once we set up experiments in a mycology laboratory with no problems. Most students have had some prior experience with sterile technique, but even if they haven't, care and common sense will yield good results. Contamination is usually visible after 7 days. It is important to use freshly-prepared bleach solution. Old diluted bleach loses its potency. We have had an occasional seed lot which was difficult to decontaminate. If you have a persistent problem with bacterial contamination try a fresh batch of seeds. The alcohol rinse is probably not essential, but we always use it. I have killed seeds by adding detergents to my bleach solution, so I no longer add any. Also prolonged exposure to bleach kills the seeds; do not exceed 30 minutes. There are two excellent plant tissue culture laboratory manuals (Dodds and Roberts, 1982; Reinert and Yeoman, 1982) which you can consult for more background information and practical advice. I would be very interested in learning how these experiments work for you, and would be happy to correspond with you or your students.

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