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CULTURE OF APICAL MERISTEMS AND EMBRYONIC SHOOTS OF PICEA ABIES APPROACH

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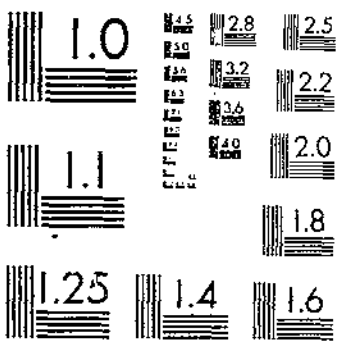

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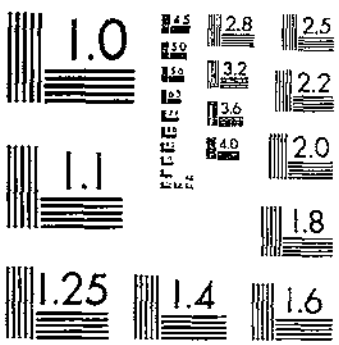

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CULTURE OF APICAL MERISTEMS AND EMBRYONIC SHOOTS of *Picea abies*—APPROACH AND TECHNIQUES

J. A. Romberger, R. J. Varnell, and C. A. Tabor

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Culture of Apical Meristems and Embryonic Shoots of *Picea abies*— Approach and Techniques

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Contents

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By J. A. Romberger, R. J. Varnell, and C. A. Tabor

INTRODUCTION

This publication describes techniques evolved during 3 years of effort directed toward axenic culture of apical meristems and embryonic shoots of coniferous trees. Some preliminary results are mentioned to illustrate the kind of growth and development which may be expected and to suggest applications of these methods to investigations of the developmental biology of woody plants.

These techniques do not have as their aim tissue culture with callus production, but organ (and eventually organism) cultures exhibiting differentiation and morphogenesis approaching the normal in type. Whereas the present procedures are not the ideal ones, they will serve as a standard of comparison and departure for later studies on physical and biochemical control of development at the shoot apex. The intent of this report is to give sufficient detail to enable others to adapt these procedures to their own research problems with a minimum of preliminary work.

There is practically no technical literature specifically dealing with the problems of culturing gymnosperm apices. The papers by Hollings (1965) and Isikawa (1967) will provide access to both the literature concerning the biology of meristems and the practical aspects of meristem culture.

The term "apical meristem" as used here refers to the smooth-surfaced apical dome itself plus any young primordia in the process of initiation around its flanks and base which have not yet surpassed the dome in height. When only the dome above the youngest primordia is intended, the term "apical dome" is used. The larger structure, encompassing apical meristem, subapical meristem, and all associated primordia or developing leaves, is called the "shoot tip" (fig. 1). "Embryonic shoot" as used here refers to all those tissues and organs present in the bud during summer, fall, and winter, which will contribute to the shoot to be expanded in spring (fig. 2). It includes all tissues and organs within the scales and above the "crown" (see Romberger 1963) or "nodal diaphragm" (Venn 1965). Therefore, "embryonic shoot" is not synonymous with "bud." In genera not having "crowns" the embryonic shoot is less distinct and must be defined less restrictively.

Figure 1.—A partially dissected shoot tip of a 4-month-old *Picea abies* seedling revealing the dome-shaped apical meristem with young primordia on its lower flanks. The smooth-surface apical dome may be excised and grown in axenic culture. The dome is about 300μ wide and 200μ tall.

Picea abies, the Norway spruce, was chosen for this work for several reasons. The buds are essentially nonresinous, and their internal structures are almost devoid of epidermal hairs. Well-defined embryonic shoots are available for about 10 months of the year, and they are sharply demarcated from older tissues by a dense nodal diaphragm in fall and winter. The most significant feature of the seedling shoot apical meristem is its smooth apical dome, 150μ to 250μ tall and 250μ to 400μ in diameter. This facilitates excision without the use of micromanipulators. Also, *P. abies* seed, which is readily available from a variety of geographic sources, can be germinated in less than a week, usually without prior cold stratification, and the seedlings tolerate a wide range of greenhouse and outdoor conditions. Finally, the literature on the physiological and botanical aspects of *P. abies*, though still inadequate, is more extensive than that concerning most other conifers. For these reasons we have concentrated our early work on *P. abies*; however, we fully realize that the behavior of other species might

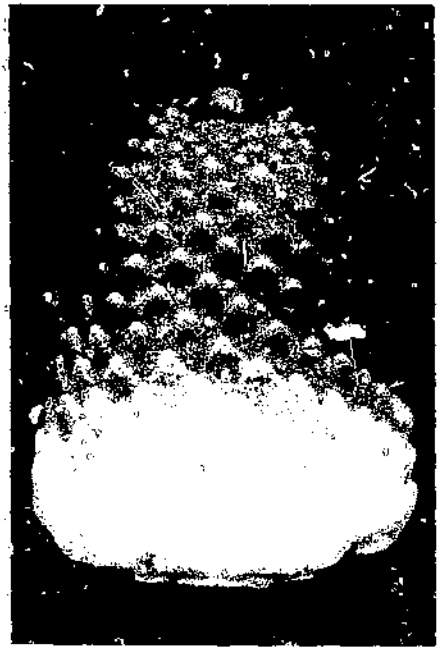

Figure 2.—A typical vegetative embryonic shoot from a dormant *Picea abies* bud collected in early winter. The excision was made just above the nodal diaphragm or "crown." Height is about 2 mm.; fresh weight, about 4 mg. Generally the primordia of all the leaves appearing on a new shoot during summer are already present in this dormant structure. A dome-like apical meristem—the origin of all else—surmounts the structure. The dome is only slightly larger than that of the seedling in figure 1.

be different and equally interesting.

Study of the in vitro growth characteristics of embryonic shoots and apical meristems requires a continuous and convenient supply of buds or shoot tips. For some experiments apical meristems or embryonic shoots collected from buds of trees growing outdoors are required. We collect these from a number of scattered, mature trees and from a plantation established about 30 years ago. Collection of large numbers of vigorous buds is, unfortunately, quite destructive to the trees. For most experiments at this stage of our studies, however, apical meristems from successive crops of seedlings grown under greenhouse conditions are preferable, and the supply is more practical to maintain.

Picea abies seedlings can be grown in the greenhouse from seed to a height of 25 to 50 cm. in 6 to 9 months in one continuous flush. During this phase the apical meristem continually initiates new primordia, and these develop into leaves without an intermediate period of rest or dormancy. The subapical meristem (the poorly defined axial meristematic zone beneath the apical dome) is also continuously active in producing new cells in the axial segments to which the primordia are attached. These activities result in continuous elongation growth. But finally, the developmental pattern of the primordia changes so that they become scales rather than leaves, and a concomitant, drastic reduction of subapical meristem activity presages formation of the first terminal bud. Present indications are that in many instances any great reduction in growth rate can trigger this developmental change. Moderate changes in the photoperiod per se probably have no special efficacy.

Changes in developmental processes at the apex are the key to many problems related to tree growth and dormancy. For example, young spruce seedlings have a continuous, open growth habit during a long, initial, budless "flush." Older trees, in contrast, exhibit a strongly episodic growth habit of rather different characteristics, in which one generation of buds gives rise to another. The change of habit accompanying the formation of the

first terminal bud offers an opportunity to apply organ culture and other techniques of developmental biology, to the problem of the control of morphogenesis in trees.

Once the first terminal bud has been formed, with an embryonic shoot sequestered within it, growth does not readily revert to the continuous pattern. Instead, formation of a new bud begins while the embryonic shoot of the preceding bud is still in its brief, spring elongation phase.[1] Embryonic shoots also offer opportunities for the study of development and morphogenesis, particularly with respect to determination and early differentiation of male and female strobili. For this reason we have done some preliminary work on the axenic culture of embryonic shoots as well as on the 500-fold, smaller apical meristems.

The large, scale-clad, vegetative bud with its enclosed embryonic shoot (fig. 2), including perhaps several hundred leaf primordia, is conspicuously present in *Picea*, *Abies*, *Pseudotsuga*, and some other genera of conifers, but there are also numerous genera in which it is lacking. *Thuja*, *Libocedrus*, *Cupressus*, *Chamaecyparis*, and *Juniperus* have no sharply defined period of elongation growth. They form no vegetative buds containing well-developed embryonic shoots, and therefore they exhibit only a weakly episodic growth. In a sense, the continuous growth habit characteristic of the spruce seedling is retained by the adults of these latter genera. Apical meristems from the small, scaleless "buds" of those members of the Cupressaceae which we have examined have all been very small. They can, nevertheless, be isolated and transferred to culture dishes by the techniques described here.

SEED SOURCES

Picea abies seed from various geographic areas in Europe and North America is available from commercial seed dealers. Though we have grown seedlings from seeds from more than a dozen sources, we are not able to predict growth behavior under our conditions on the basis of seed source. The growth behavior of seedlings from seeds of geographically contiguous European regions has sometimes been quite different.

A common problem in greenhouse culture of *Picea* is undesirably early formation of the first terminal bud, ending the continuous-growth phase of the seedling prematurely. This is particularly likely to occur during or after periods of low-light intensity, and it cannot always be effectively prevented by artificially extended photoperiods or by supplemental daytime illumination of the 300- to 400-ft.-c. intensity normally available in greenhouses. Present indications are that some subarctic provenances of spruce are rather tolerant of low-light intensity and are therefore easier to maintain in the actively growing condition in greenhouses during fall and winter. Seedlings from such provenances may, nonetheless, be undesirable because of generally slow growth. Seed from the Austrian Boehmerwald has been fairly satisfactory under our greenhouse conditions. Seed is stored at 2° to 3° C. in screwcap jars also containing calcium sulfate or silica gel desiccant. We have not demonstrated that such cold-dry storage is beneficial.

[1] For a discussion of the morphogenic cycle of *Picea abies* in terms of contemporary concepts of developmental biology, see Romberger (1966a).

SEED GERMINATION

Seed is germinated in a small controlled-environment plant-growth chamber. Temperature is maintained at 27°±1° C. during the 12-hour photoperiods and at 18°±1° C. during the 12-hour dark periods. Light intensity at the substrate surface during photoperiods is about 800 ft.-c., with 700 ft.-c. being provided by cool-white type fluorescent tubes and 100 ft.-c. by incandescent lamps.

Seed is sown on a 9-cm.-deep bed of milled horticultural sphagnum moistened with nutrient solution as described under greenhouse procedures below. The seed is covered with a double layer of bleached cheesecloth, which is removed when hypocotyl elongation begins 4 or 5 days after planting. The seedlings are ready for transplanting to greenhouse flats 10 days after planting when hypocotyls are about 3 cm. long and growing at a declining rate. Radicles at that time are 4 to 7 cm. long and fairly straight. Enough seed is planted to provide a 50- to 100-percent excess over the required number of seedlings. Only the more vigorous individuals are transplanted.

GREENHOUSE PROCEDURES

Seedlings are grown in a greenhouse equipped with thermostically controlled vents and steam-heated convectors. During the winter this allows temperature control to within about ±2° C. of the desired mean value. During winter the day thermostat is set at 21° C., and the night thermostat, at 15° C. Temperature control during the summer is less satisfactory. A modest degree of control is provided by an evaporative cooling system. The efficiency of this system varies with the outside relative humidity. Except on the hottest, most humid days, inside temperature can be maintained appreciably lower than outside, sometimes 6° C. lower. Stripes of white shading compound covering about 60 percent of the glass surface are applied in spring and removed in fall. This reduces inside light intensity to about half the outside value.

As a routine practice, incandescent illumination of about 350 ft.-c. intensity is provided in the morning and evening to extend the photoperiod to 18 hours from early September through April. The efficacy of this practice in itself in maintaining growth of seedlings in winter has not been established, but it does eliminate the photoperiod as a variable during the winter.

Seedlings are grown in 14- by 20- by 3.5-inch wooden flats, 48 plants per flat. Wood slats are put between the flats and tabletops to alleviate drainage problems. As a rooting medium, prepared "potting soil" is more expensive and less satisfactory than milled horticultural sphagnum (not peat) moss. The latter is shipped dry and weighs less than 4 pounds per bushel. Sphagnum eliminates damping-off diseases and also simplifies moisture regulation. Also, the use of nutrient solutions to wet the sphagnum permits repeated reproduction of nutrient regimes more readily and exactly than is possible with soil mixtures.

The formulation of a nutrient solution with which to wet the sphagnum was somewhat arbitrary, although we took advantage of available information concerning nutrition of seedlings of *Picea* and other conifers (Němec 1938; Leyton 1952; Ingestad 1959, 1962;

Pharis, et al. 1964). The addition of nutrient solution to dry sphagnum to make a rooting medium is not comparable to fertilizing soil. In the sphagnum mixture the largely immobilized water constitutes a major percentage of the weight and nongas volume of the total medium and is not closely comparable to the so-called soil solution of a soil system. Although the total mineral content of sphagnum is low, the cation-exchange capacity of its organic colloids is relatively high (see Brehm 1968), and new supplies of nutrient elements become available to growing root tips as they advance through the medium. For these and other reasons, the wet sphagnum medium is also not comparable to a culture system in which roots grow in an aerated nutrient solution.

Because the sphagnum medium is comparable to neither water solutions nor soil, we could not justify the adoption of fertilization procedures intended for soil or water culture systems. The formula given in table 1 was adopted only on the basis of adequate performance and is not presented as an optimal mixture. It is similar to that arrived at by Ingestad (1959) on the basis of growth of *Picea* seedlings under low light intensity in water solutions, but it is considerably more concentrated and contains a high level of urea rather than inorganic nitrogen (see Pharis, et al. 1964). The moderately high chloride level of the solution is of no immediate concern because the sphagnum is originally low in chloride, and Themlitz (1966) has shown that, for *Pinus sylvestris* seedlings at least, chloride-based fertilizers perform just as well as equivalent sulfate-based formulations.

TABLE 1.—*Composition of nutrient solution used for wetting sphagnum*

Macronutrients:

Component	G./l.	Concentration
Urea	1.075	1.79×10^{-2}M
$MgSO_4 \cdot 7H_2O$	.770	3.12×10^{-3}M
$CaCl_2 \cdot 2H_2O$	.735	5.0×10^{-3}M
KCl	.357	4.80×10^{-3}M
KH_2PO_4	.220	1.61×10^{-3}M
$FeCl_3 \cdot 6H_2O$	.025	9.1×10^{-5}M

Micronutrients prepared and stored as 10,000 × stock solution:

Component	G./l. stock solution	
H_3BO_3	14.30	Add 0.1 ml. of this stock solution per liter of macronutrient solution.
$MnSO_4 \cdot H_2O$	7.82	
$ZnSO_4 \cdot H_2O$	1.37	
$CuSO_4 \cdot 5H_2O$	.590	
$CoCl_2 \cdot 6H_2O$	.405	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	.185	

Elemental composition:

Major elements		Minor elements	
Element	P.p.m.	Elements	P.p.m.
N	500	B	0.25
P	50	Mn	.25
K	250	Zn	.05
Ca	200	Cu	.015
Mg	76	Mo	.010
S	100	Co	.010
Cl	534		
Fe	5.2		

To insure against micronutrient deficiencies common in sphagnum and peat, and possible associated aberrant meristem behavior, the nutrient solution is routinely fortified with a modified "Hoagland's" (Hoagland and Arnon 1938) trace-element solution supplemented with cobalt chloride. This is, however, only insurance against an unsuspected deficiency, and we have no evidence that growth rates are affected by such additions.

Because of the large volumes required, solutions for greenhouse use are made up with tapwater, and tapwater is also used for all subsequent watering. Flats are watered sufficiently to compensate for evapotranspiration and to prevent surface salt accumulation, but not so generously as to promote leaching. (Weight checks readily reveal excessive or insufficient watering.) Initial pH of the sphagnum wet with nutrient solution is 3.8 to 4.0. This rises gradually to 4.5 or slightly higher during a 2-month growing period.

In practice, nutrient solution and milled sphagnum are mixed at the rate of 2.36 liters of solution per kilogram of dry sphagnum (15 liters per nominal 2-bushel, approximately 3.5-kilogram, sack). This mixture provides a desirable working consistency. The wet sphagnum is then packed into 14- by 20- by 3.75-inch flats with moderate compaction so that the yield is about two and one-half flats per sack of sphagnum.

Excessive initial compaction combined with generous subsequent watering leads to inadequate subsurface aeration. When this occurs, the "fermenting" sphagnum turns lighter in color than sphagnum having access to oxygen and also emits a foul odor. Normally *Picea* seedlings grow just as well, or better, in a continuous bed of sphagnum in a flat as they do when planted with the same spacing in sphagnum-filled peat pots set on a 1-inch bed of sphagnum in similar flats. The seemingly desirable airspaces between pots do not result in better growth. Though we initially grew all seedlings in peat pots, we do so now only when experiments require individually potted plants.

Transplanting from germination flats to greenhouse flats (48 seedlings in each) is best done quite early—while most seedcoats are still capped over the cotyledons. Large, vigorous seedlings from the early germinating seeds are transplanted. Late germinators and slow growers are discarded. In planting, spacing is marked out with a jig, and holes are made with a pencil. The radicle of a selected seedling is inserted into a hole, and the sphagnum is pressed into firm contact with it. If the last step is slighted, the growing roots tend to push up the seedlings rather than to penetrate the sphagnum themselves.

Immediately after planting and moderate watering, the surface of each flat is sprinkled with enough coarse-white-quartz sand to just cover the sphagnum. This serves several purposes. Most importantly it greatly increases the reflectivity of the surface and significantly increases the effective light intensity on the leaves. This is especially important in winter and during cloudy weather. Also, the sand's high reflectivity is desirable during sunny summer days when it helps keep the rooting medium cooler. In addition the sand reduces the surface growth of algae and the local accumulation of salts due to wick action.

Sphagnum apparently contains eggs of the small black fly, *Scatella stagnalis*. When conditions are favorable, these may hatch into small, white larvae which quickly develop into adults. We have no evidence that larvae or adults seriously damage spruce seedlings. Rotenone used to control aphids also controls these flies. We have avoided the use of other pesticides. Autoclaving sphagnum to control potential pests is not recommended. Autoclaving sharply increases the pH, destroys the normal microflora, promotes vigorous colonization by undesirable organisms, and results in poor seedling growth.

Growth rates of seedlings vary with seed source and with season of the year, but are normally between 3 and 5 cm. per month. Seedlings are a convenient source of apical meristems after they have made about 4 cm. of epicotyl growth. Meristems from smaller seedlings are more difficult to excise because of their smaller size, but they grow well when cultured.

DISSECTING TOOLS

The usual dissecting scalpels available from laboratory supply houses are much too large to be useful in the final stages of isolation of an apical meristem or even of a whole embryonic shoot. Semimicroscalpels of greater utility can be made by shaping and sharpening certain types of dissecting needles. The commercially available half-spear "needle" with tapered aluminum handle has been very useful, particularly in the isolation of embryonic shoots from buds. It has a single cutting edge about 7 mm. long and a very acute tip. The size can be reduced by grinding.

The angle handpiece of a miniature, flexible-shaft machine clamped under a stereo dissecting microscope facilitates precision grinding and sharpening of such tools. A portable dental engine can also be used. A variety of burs and grinding wheels are available from the manufacturers of such machines. In our experience, however, even the finest grained miniature grinding wheels are too coarse for sharpening microdissecting tools. We routinely cement a "French emery" paper disk onto the wheel before use. Such disks are made in various types and sizes for dental use and are available from dental supply houses. Final honing is best done without use of power tools. Small Arkansas stone "slips," rouge paper (both obtainable from jeweler's supply houses), and pieces of leather cut from a barber's strop are useful in finishing.

Though suitable for working with buds and excising embryonic shoots, the scalpels described above are still comparatively large. Blades 20 to 50 times smaller are needed to enable one to work effectively with apical meristems. Razor-blade fragments offer some possibility, and we have sometimes used them with modest success. They are, however, difficult to break or grind to the exact shape desired and are also difficult to mount. Furthermore, the fractured edges of some types rust easily. In our experience, watchmakers' flat pivot drills are a more useful and versatile starting material than razor blades for making microscalpels.

Many sizes and types of microminiature drills of high-speed tool steel are made for the watchmaking industry. Flat-type pivot drills [2] are most suitable for micro-

[2] Such drills are available from major watchmakers' and jewelers' supply houses.

tool making. These are available in about 100 sizes (blade width) between 40μ and 1 mm. Sizes between 200μ and 300μ are most generally suitable for making microscalpels for meristem work (fig. 3). The integral shanks of the drills are sufficiently long and thick to be held in adjustable-chuck dissecting needle holders which we have made into microtool handles (fig. 4).[3]

Making microscalpels from 200μ-300μ-pivot drills is simple in theory but moderately demanding in practice. It is advisable to begin with a fairly large size (about 500μ) to gain experience before attempting to make smaller blades.

Rough shaping is conveniently done with the aid of a small flexible-shaft machine. Clamp the handpiece of the machine in such a manner that the grinding wheel (with a fine French emery disk cemented to its upper surface) is held horizontally just above the stage of a stereo dissecting microscope, and so that the working end of the handpiece exerts some downward pressure upon a small rubber block under it. This will reduce vibration. In grinding, use a high

Figure 3.—A 250μ flat pivot drill and a microscalpel made from such a drill.

shaft speed and the lightest possible working pressure. Wipe the blade frequently on a wet piece of thick filter paper to remove grinding debris and to improve visibility of the edge. Considerable care is required to produce a blade with optimal shape, strength, and cutting edge. At first some failures must be expected. Later a several months' supply of microscalpels can be produced in a few hours.

Final shaping, sharpening, and polishing can be done by several different methods (all under the microscope, of course). A fine-grained Arkansas stone with light oil can be used for final grinding, and then finishing and polishing can be done on rouge paper and leather. However, better results are usually obtained with a bit of diamond dust in a film of light oil on the carefully prepared (with a fine-toothed mill file) end grain of a boxwood slip (fig. 5), followed by rouge paper (the latter is cemented to a small stick of wood for con-

[3] The Arthur H. Thomas Company's No. 4607 needle holder can easily be modified to make an inexpensive, comfortable microtool handle. The handle is lengthened to provide better balance and control, and a larger diameter rubber grip is added so that the handle can be held like a pencil. A simple method of lengthening the handle is to slip a piece of hot-water-treated, snugly fitting, semi-rigid, polyethylene tubing over the entire length of the original handle, leaving about 2 inches of tubing projecting at the upper end. Then push a brass rod into the bore of the projecting tubing until it touches the end of the original handle. Cut to the length which gives the best feel and balance. A 3-cm. length of rubber tubing positioned over the polyethylene tubing just above the chuck forms a suitable grip.

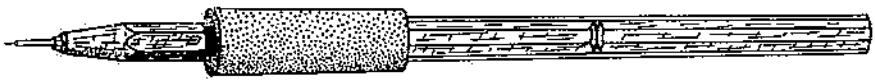

Figure 4.—Microtool handle made from an adjustable-chuck dissecting needle holder.

venience in holding under the microscope). The materials mentioned are available from jewelers' suppliers. Final cleaning is done on wet filter paper. Blade surfaces should have almost mirror smoothness. The latter seems to be almost as important as edge sharpness in preventing widespread cell damage during excision of and microsurgery on apical meristems.

In addition to microscalpels, microforks also are useful in handling meristems. These can be made from small stainless steel hypodermic needles. The method has been described elsewhere (Romberger 1966b).

Microtools can be used to their best advantage only if the plant material can be securely and conveniently held under the microscope. Precision-made, angle-tipped, ultrafine pointed, microdissecting forceps are needed for this.

STERILIZATION OF DISSECTING TOOLS

Flame sterilization of microdissecting tools is unsuitable because of loss of temper and oxidation of the metal. Heat sterilization, nevertheless, is desirable because it is thorough and chemically noncontaminating. Fortunately, heat sterilization can be accomplished quickly and effectively. The heat source is a cheap pencil-type electric soldering iron having a power consumption of about 45 watts and a maximum temperature of about 450° C. This tool is clamped upright near the dissecting microscope. The regular soldering tip is removed and replaced with a piece of snugly fitting stainless steel tubing 25 to 30 mm. long. The upper end of the tube has been flattened and bent 45° to one side to form an inclined table about 8 by 12 mm. (fig. 6).

A microtool is sterilized by wiping it carefully on wet filter paper, dipping it in alcohol, and then holding the shank of the tool onto the hot table for a few seconds. The blade of the tool does not touch the table; instead, it is heated by conduction. The tool, having a very small mass, cools to a safe working temperature within a few seconds. Because carbonized organic matter or baked-on protein is difficult to

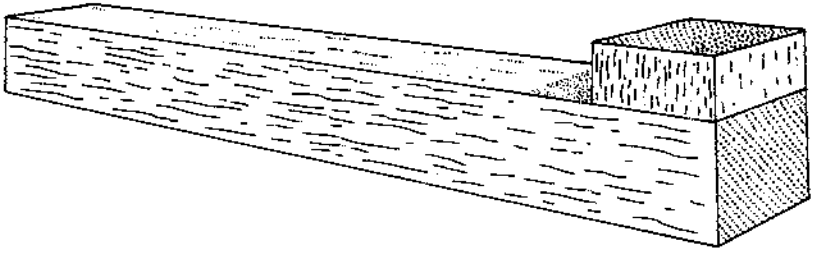

Figure 5.—The end grain of a boxwood (*Buxus* spp.) block (6 mm^2) is used for shaping and sharpening microscalpels under the microscope. The abrasive is fine diamond dust suspended in light oil.

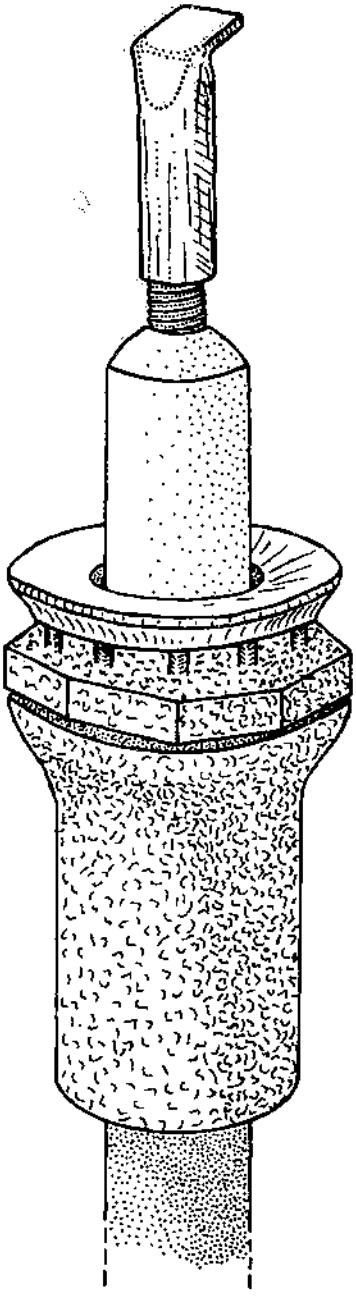

Figure 6.—Heat sterilization "table" made by replacing a soldering iron tip with a flattened and bent stainless steel tube.

remove without damaging the blade, it is important that the blade be wiped clean before each heat sterilization.

STERILITY DURING DISSECTION

Embryonic shoots from buds and apical meristems of growing seedlings are evidently sterile in their natural state; or at least, they do not carry micro-organisms which can form microscopically visible colonies on simple mineral salt, sugar, and agar media (see Ball 1946, Keener 1951). Before accepting this as reliable fact, we assumed the necessity of surface sterilization prior to dissection. However, no sterilization method tried was without harmful effects upon the tissue. We had no success until we changed our approach to that of maintaining natural sterility. We now make no effort to sterilize contaminated outer appendages of buds or growing shoot tips. When we remove these appendages, we think of them as contaminated and sterilize our tools frequently while proceeding inward. When the embryonic shoot or apical meristem becomes accessible, it must be handled aseptically, but it need not be given any antiseptic treatment. The fact that tissue-borne contamination has been exceedingly rare in our laboratory confirms our belief that in this work meristematic tissue sterilization is unnecessary.

DISSECTING TECHNIQUES

Excising apical meristems from seedlings or isolating embryonic shoots from buds requires techniques which, though mechanically different, are similar in certain requirements. Sterility of internal

structures must be maintained; desiccation of succulent inner tissues must be prevented; and the parts to be isolated for experiments must be handled only in the most deliberate, careful way to avoid mechanical injury. Such injury can result in gelation of the cytoplasm (microscopically recognizable by a change in appearance of cells from near transparency to a whitish translucency) or loss of turgor and cellular collapse.

Excision of apical meristems is rather demanding. Satisfactory volumes of high-quality work can be expected only if some attention is paid to the comfort and convenience of the operator as well as to maintaining the tissue in an uncontaminated, undamaged condition. An adjustable-magnification, stereo-dissecting microscope with a pedal-operated electric focusing device is a convenience. Lamps must be intense and focusable. Radiant heat must be removed from light beams by heat-absorbing filters.

Whereas an embryonic shoot can be isolated by removal of bud scales, by working inward until the green "berrylike" structure becomes accessible, this is excruciatingly laborious and generally unsatisfactory. The following procedure offers several important advantages:

From a 7- to 10-mm.-thick aluminum plate, prepare a small block about 12 mm. wide and 35 mm. long. Taper one end of the block slightly so that it can easily be grasped with a moderately large forceps (fig. 7). Drill a row of holes through the block, using four or five drills with a range of diameters similar to, but somewhat smaller than, that of the buds from which you wish to isolate embryonic shoots. Ream the holes slightly, and put a smooth finish on all surfaces of the block. The block will serve as a jig to hold buds, apex down, under the microscope.

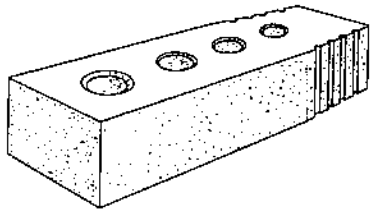

Figure 7.—A jig for holding buds during dissection. It was made from 0.25-inch-thick aluminum plate.

Begin the dissection by cutting the bud from the twig with enough basal tissue so that all scales are included. Then, working on a piece of sterile filter paper (high wet-strength type), use a small scalpel to peel off a few outer layers of scales. Employ an "unrolling" technique so that the bud is continuously rolled onto clean, sterile paper away from the scales being removed. While many whitish, translucent inner scales still remain, use a sterile forceps to place the bud, point down, in a suitable hole in the jig under the microscope. Using a small, very sharp scalpel (such as the sharpened half-spear needle mentioned earlier), begin cutting off cross sectional layers from the base of the bud. When you approach the nodal diaphragm (or crown), the color and texture of the tissue in the pith region become noticeably different. Work carefully, sterilize tools frequently, and expose the crown.

Next, prepare to cut along the morphologically upper surface of the crown with a sharp, sterile blade. This will cut away the axial attachments of the remaining scales and leave the embryonic shoot lying free in a cavity formed by the encasing capsule of bud scales. It can be made to pop up by exerting a slight lateral pressure on the enclosing scale structure. The freshly cut basal surface of the embryonic shoot will usually be moist enough to adhere to the side of a blade; if it does not, push the tip of a microscalpel between some basal primordia until the structure can be lifted. The embryonic shoot should be transferred to a culture plate or other moist surface with a minimum of handling and exposure.

Somewhat more demanding than isolation of an embryonic shoot is the excision of an apical dome in so gentle a manner that it will con-

tinue growth and development in culture. We have used the procedure described below to isolate about 4,000 apical domes, mostly from *Picea abies* seedlings:

Soak some 7- or 9-cm. circles of high wet-strength filter paper in distilled water in a large Petri dish. Drain, leaving a slight excess of water, and autoclave. Collect shoot tips from seedlings only when you are actually ready to begin dissecting. Keep them in a covered Petri dish on wet paper.

The initial step in the isolation of the apical meristem can be done with the unaided fingers by pulling or breaking off all leaves which can easily be grasped. Enough leaves will remain to protect the apex. Cut away excess stem length, leaving only enough to allow firm holding with a sterilized, angle-tipped microforceps. Transfer the stem tip to a disk of the above-mentioned autoclaved paper lying on the microscope stage. The paper must be wet enough so that it is held flat on the stage by surface tension forces and does not slide easily.

While holding a 200μ to 300μ microscalpel as you would hold a pencil, remove successively smaller leaves and primordia until the apical dome is exposed. An optical system allowing gradual increase of magnification is advantageous for this work. It is not necessary to cut off each appendage. Many of the larger ones will snap off readily when bent away from the axis. Wipe and sterilize tools frequently. It is usually sufficient to expose the apical meristem from the upper side only (as in fig. 1), leaving the appendages on the lower side to give mechanical support when the final severing cuts are made. Now adjust the position of the shoot and the focus of the microscope so that you can clearly see the apical dome in profile. Decide where you want to cut, and clear away any remaining tissue that might interfere with the precisely controlled movements of the scalpel. Carefully wipe and sterilize the microscalpel to be used for making the critical final cut. A "pencil hold" of the microscalpel is unsuitable for this operation. It is better to grasp the rubber grip of the holder between the pads of the thumb and forefinger with the handle extending under the palm (not up between the thumb and forefinger). Rest the hand on the stage while thus holding the microscalpel. Only very slight motion is possible without moving the whole hand, but that slight motion is sufficient, and it can be precisely controlled.

Make the final severing cut in several downward passes, using the slender tip of the scalpel. Advance the tip about 50μ from right to left between downward strokes. When the apical meristem has been completely severed, it will readily adhere to the scalpel, and it can be transferred to a clean area on the moist paper surface. Any necessary trimming away of adhering primordia or other tissue fragments is best done at this stage. Removal of the meristem from the paper to the culture plate can be done with a microscalpel, microfork, or microspatula. But any transfer through air of such a small piece of tissue must be done very quickly, or drying protein may cement the tissue to the tool so that it cannot easily be removed.

When it is necessary to isolate apical domes from embryonic shoots, the latter are first isolated from buds as already described. The embryonic shoots are then held with a microforceps in such a position that the apical dome can be cut off with a microscalpel.

Tissue-borne contamination has been very rare when the above techniques have been used. Airborne contamination, however, can still be a problem. We have almost eliminated this hazard also by careful dust wiping in the laboratory, sealing the windows, and regularly cleaning the air filters in the heating and air-conditioning system.

CULTURE VESSELS

We have not yet found a source of supply of what would seem to be the ideal culture vessel—a factory-sterilized, disposable-plastic, thimble-sized cup with a deep-lipped cover. As the best available substitute for the ideal, we have been using 35- by 10-mm. plastic Petri dishes; these conveniently contain

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about 3.5 ml. of agar medium. Seven of these small dishes fit around the inside periphery of a 6-inch glass Petri dish. We thus find it convenient to work with sets of seven meristems. Each meristem is cultured inside its own small dish, and all seven of the latter are kept inside a single large dish.

CULTURE CONDITIONS

Some growth and development of meristems in culture can be obtained under a wide variety of temperature and light conditions (or in darkness). For a serious experimental work, of course, some set of standard conditions must be established as a basis against which to evaluate effects of deviations from it. We routinely incubate all cultures in small plant growth chambers. Illumination of 250 ft.-c. at culture level is provided by cool-white fluorescent tubes supplemented, when desired, by an additional 75 ft.-c. from tubular incandescent lamps. Temperature is controlled at 25±1° C. during the 14-hour photoperiod and at 21±1° C. during the 10-hour dark period. These conditions are arbitrary standards, and the values selected have no demonstrated special significance.

The possibility that day-to-day changes in the composition (including pollutants) of the atmosphere have an effect upon growth of cultures cannot be discounted. We, nevertheless, accept these and other minor, unregulated environmental parameters as variables which affect treatments and controls alike. High humidity is maintained, as described below, for the purpose of minimizing evaporation from the culture media.

MINIMIZING MOISTURE LOSS

Relative humidity within the 6-inch Petri dishes containing the small culture dishes must be kept high; otherwise, moisture loss during the 3-week culture periods will be excessive. A 55-mm. dish of water in the center of the large Petri dish is a suitable moisture reservoir.[4]

In addition to keeping a moisture reservoir within each 6-inch Petri dish, it is advisable to place these dishes in close-fitting trays to retard air movement across the gap between lid and bottom. The dishlike cardboard trays we use for this purpose further reduce contamination rates as well as water loss. Relative humidity within the entire incubation chamber is kept high by placement of trays of water on the bottom near the circulating fan. Under this combination of conditions cumulative 3-week moisture losses are 20 to 22 percent of the initial medium volume.

[4] We formerly placed a small, water-saturated block of regenerated cellulose sponge in the center of each large dish. This procedure, however, is not recommended. Commercial cellulose sponges frequently contain urea as a residue from one of the final manufacturing operations. After 5 to 10 days of water saturation, microbial populations on and in the sponges will be hydrolyzing any urea present and ammonia will be evolved. The ammonia gradually diffuses into the culture dishes, dissolves in the medium, and may cause the pH to rise to lethal levels. Autoclaving the sponges does not solve the problem because the heat and moisture hydrolyze the urea directly, and a new microflora develops quickly. Under otherwise identical conditions, pH changes do not occur if the sponge blocks are replaced by some other moisture reservoir. Our analysis of this "pH problem" has been confirmed by correspondence with two major cellulose sponge manufacturers. The sponges are, presumably, safe to use after thorough leaching.

FORMULATION, PREPARATION, AND HANDLING OF CULTURE MEDIA

APPROACH

How one chooses to approach the problem of formulating a medium suitable for the culture of embryonic shoots or of apical meristems depends largely upon one's goals. Our goal is not limited to achieving the successful culture of shoot apices. It includes the more distant end of learning how the environmental parameters impinging upon the meristem, the physiological mechanisms within the meristem itself, and substances normally contributed by the remainder of the tree interact to control growth and development of the meristem and the young primordia subjacent to it.

In the drive toward this goal, the first need is for a minimal basal culture medium—a sustaining medium—the most simple formulation which will still allow excised meristems to survive for a few weeks and to make whatever growth is necessary for maintenance of the meristematic potential. If the basal medium already contains, by design or as incidental components, an array of metabolites, vitamins, or substances having hormonal or growth regulator activity, then it becomes difficult to determine the essentiality or physiological effects of any such substances added experimentally. However, the formulation of a "minimal" basal medium is itself a problem requiring considerable experimental work.

This is true because available information concerning the behavior of apical meristems in culture is very scant. It is so meager that there is little guidance as to how one should interpret the frequently poor growth of cultures on newly formulated media. It could be due to lack of certain components essential to normal development, or it might result from the presence of substances which have deleterious effects upon growth and development. Furthermore, if deleterious effects are noted, it is not easy to learn whether they are attributable to unsuspected properties of known constituents of the formula or to substances only incidentally present as impurities or contaminants. This difficulty is further compounded by the possibility that unsatisfactory growth may result from a choice of unsuitable enviromental conditions for the cultures, or from the inadequacies of handling techniques. There is no easy solution to the problem.

We chose not to adopt a formulation from the literature. All the published formulations were designed for other plant materials and were oriented toward objectives different from ours. We have also refrained from the inclusion of coconut water, yeast extract, casein hydrolyzate, and similar chemically undefined preparations. Despite studied consideration of the experiences and results of others who have cultured gymnosperm tissues or organs (notably Ball 1950; Reinert and White 1956; Al-Talib and Torrey 1959; Steinhart, Standifer, and Skoog 1961; and Risser and White 1964), to whom we recognize our indebtedness, our choice of a "point of departure" medium was still somewhat arbitrary. Fortunately, this first medium was sufficiently innocuous to *Picea* meristems to allow meaningful tests. It was subjected to 3 years of experimentation during which time the effects of various procedural modifications, deletions, and supplements were tested in a preliminary way. We

thus finally arrived at the formulation which is given in table 2 as the basal medium. This is in no sense an end. It is merely a defined beginning.

FORMULATION AND CHARACTERISTICS

The medium prepared from the formula given in table 2 has several important advantages. It can easily be prepared from six stock solutions and two dry ingredients. Four of the stock solutions (A_1, A_2, B, and C), each containing only compatible inorganic ions, are stable and can be stored many months in the cold with no precipitation problems. The two stock solutions of organic supplements (D and E) can be conveniently stored frozen. The pH can be adjusted to a desired value (5.5±.1 in our work) within the range of 5 to 6 by adding small, predetermined volumes of a standard alkali solution. If potassium hydroxide is used, the relative amount of potassium added during pH

TABLE 2.—*Composition of basal medium for Picea shoot meristem culture*

Component	Final concentration	Mg./l. final	Mg./l. 10X stock solution
Group A (macronutrients less calcium and iron):			
Group A_1:			
KH_2PO_4	1 m.M. (1×10^{-3}M)	170. 2	1, 702.
KCl	2 m.M. (2×10^{-3}M)	149. 2	1, 492.
NaCl	0. 04 m.M. (4×10^{-5}M)	2. 34	23. 4
Group A_2: $MgSO_4 \cdot 7H_2O$	0. 5 m.M. (5×10^{-4}M)	123. 25	1, 232. 5
Group B (calcium and iron):			
$Ca(NO_3)_2 \cdot 4H_2O$	2 m.M. (2×10^{-3}M)	472. 4	4, 724.
$FeCl_3 \cdot 6H_2O$	0. 05 m.M. (5×10^{-5}M)	13. 5	135.
			Mg./l. 100X stock solution
Group C (micronutrients):			
$MnSO_4 \cdot H_2O$	10μM. (1×10^{-5}M)	1. 69	169.
KI	1μM. (1×10^{-6}M)	. 166	16. 6
$ZnSO_4 \cdot 7H_2O$	1μM. (1×10^{-6}M)	. 287	28. 7
H_3BO_3	2μM. (2×10^{-6}M)	. 123	12. 3
$CuSO_4 \cdot 5H_2O$	1μM. (1×10^{-6}M)	. 250	25. 0
$Na_2MoO_4 \cdot 2H_2O$	1μM. (1×10^{-6}M)	. 242	24. 2
H_2SO_4 (concentrated)			0. 05 ml.
			Mg./l. 200X stock solution
Group D[1] (organic supplements):			
myo-Inositol	0. 05 m.M. (5×10^{-4}M)	90. 1	18, 020.
Thiamin·HCl	0. 5μM. (5×10^{-7}M)	. 17	34. 0
Group E (organic nitrogen source): Urea	5 m.M. (5×10^{-3}M)	300. 5	60, 100.
Carbon source: Sucrose	0. 1 M	34, 230	
Gelling Agent: Agar[2]			1. 250 or 2. 500 g./l.

[1] Stock solutions of groups D and E (both 200X) are stored frozen and thawed when needed. Equal aliquots of each are mixed and sterilized by cold filtration (becoming 100X by mutual dilution). The required amount is added to the autoclaved medium 1 min. before dispensing begins.

[2] "Purified" grade agar is used at a final concentration of 0.125 or 0.25 percent.

adjustment is so small that the percentage change in total K^+ concentration is negligible.

Use of sodium hydroxide can also be justified, particularly in experiments involving several agar concentrations. Even highly purified commercial agar preparations contain about 0.1 percent sodium (supplier's assay). Thus, the agar is a major source of sodium in our medium. Agar has a slight alkalizing effect upon medium pH; therefore, as agar concentrations are reduced, more alkali is needed to restore the pH to a standard value. If sodium hydroxide is used, it will partially compensate for the decreased sodium contribution by the agar. In all cases precautions must be taken to keep the carbonate content of the alkali low; otherwise, precipitation problems will arise.

The pH of poured agar plates of this medium, if protected from ammonia and acid fumes, microbial contamination, and water loss, remains stable for 6 months or more. Agar plates of this medium also show no tendency to develop precipitates in storage, although the same mixture of solutions, in the absence of agar, yields an iron-containing floc within the first few hours after autoclaving.

The concentrations of sucrose, urea, inositol, calcium nitrate, or other components here specified may not be optimal, but this medium will allow sufficient growth and development of excised meristems to permit meaningful tests. The actual amount of growth, however, depens heavily upon the type and concentration of agar used to gel the medium. Though not primarily a nutrient or carbon source, agar is also not completely inert. It is, furthermore, a major component of the mixture and is perhaps the least pure and least rigorously defined chemically. Both agar quality and agar concentration have very considerable effects on meristem growth. These are discussed later. It is, however, emphasized here that experiments concerning the effects of a test substance added to the medium can have little validity if growth of the cultures is being determined by properties or constituents of agar gels.

PREPARATION AND STORAGE OF MEDIA

For several reasons, it is desirable to prepare the plates of culture medium at least a week before they are to be used in experiments. This allows time for any contaminated plates to be detected and discarded. Equally important, it allows the new gel system to approach an equilibrium solvation state. During the first few days after preparation, some modifications of the basal medium may, under some conditions, undergo considerable syneresis or desolvation (particularly when disturbed) so that droplets of liquid accumulate on the surface. By the end of a week, most of the excess liquid has been reimbibed or lost by evaporation. Further changes of this kind are slow and unimportant.

Predictable reproducible results are obtained if the various ingredients of the medium are added in a specified order. For example, if the desired volume is 250 ml., 150 ml. of water is put into a 250-ml. volumetric flask. This is followed by 25 ml. of A_1, 25 ml. of A_2, 0.2 to 0.6 ml. of 0.1 N standard alkali (the exact amount required to assure that the medium will be at the desired pH after cooling varies with the agar and other ingredients and must be predetermined by titration during trial runs), 25 ml. of B, and 2.5 ml.

of C. The volume is then brought to 250 ml. with water. This mixture is added to a dry 1-liter conical flask already containing the specified amounts of sugar and agar. Autoclaving at 120° C. for 15 minutes follows. Delay must be avoided after B and C are added.[5]

After the flask is removed from the autoclave, it is immediately set on a folded cloth towel on the lab bench (room temperature 23° ±1° C.) and allowed to cool for 14 minutes. (The temperature will then be about 72° C.) Meanwhile, 10 to 20 ml. of an equal-volume mixture of solutions D (*myo*inositol and thiamin) and E (urea) has been cold-sterilized by filtration through a cellulose polyacetate membrane filter (0.22μ pore size). The latter was previously leached with boiling distilled water to remove glycerol or other conditioning agents. After 14 minutes, a 2.5-ml. aliquot of the sterile D and E mixture is added (the 200X stock solutions, of course, becoming 100X by mutual dilution), the flask is swirled, and the medium is poured into a sterile 250-ml. buret so that dispensing can begin 15 minutes after removal of the medium from the autoclave. About 3.5 ml. of medium is put into each culture dish, and it is set aside to cool undisturbed. Temperature at the beginning of dispensing is about 62° C. and will have declined to about 53° C. in another 10 minutes. Under these time and temperature conditions, the hydrolysis of urea and other possible heat effects on media components are minimized.

It is usually convenient and efficient to prepare many plates of each specific medium preparation and to store them for later use in a series of related experiments. One should consider possible "aging" effects when this is done, although, in our experience, none have been evident. For storage of several weeks or months, the dishes may be stacked in groups of seven to 10 and rolled like coins in "Parafilm" or similar material which is first wiped with ethanol. The lap should be sealed against moisture loss with pressure-sensitive tape. This method is quite convenient if the agar gel is firm enough to allow tipping of the dishes. If the agar concentration is lower than 0.25 percent, the dishes must be kept flat and handled with great care. Consequently, wrapping of stacks is more difficult. It is, nonetheless, possible and is recommended. This storage method has been quite successful and permits great flexibility in designing experiments involving a variety of media.

AGAR EFFECTS

AGAR QUALITY

Agar quality, in the sense of commercial "grade" and "purity" level, has a marked effect upon growth of apical meristems in culture, as

[5] Solution B is prepared by dissolving calcium nitrate and ferric chloride in water. Initially the color is pale yellow, but it becomes clear reddish-brown after an hour or so. This well-known color change is generally assumed to be due to the formation of a ferric hydroxide sol. This colloidal solution is stable for long periods if not subjected to vigorous heating or to strong solutions of certain salts. The solution may be flocculated by autoclaving in the absence of a protective colloid such as agar, but a small amount of agar in solution gives protection. The agar can, of course, be dissolved in the water by preliminary heating so that its protective action can be fully utilized but it is usually equally satisfactory and more convenient to add B and C to the other ingredients immediately before autoclaving. The agar will dissolve quickly enough to give protection, and no precipitation or flocculation problems will usually arise.

expressed by dry-weight increases. In beginning meristem culture work, we used Difco Laboratories Special Agar, Noble, for all experiments. This is a "washed" agar widely used in water testing, in immunodiffusion and electrophoresis techniques, and sometimes in tissue culture work (White and Risser 1964). The advent of commercially purified agars a few years ago prompted us to test their performance against a group of bacteriological and special-purpose agars. Large differences were quickly evident. Details of this work will be published elsewhere. The results may be summarized as follows:

Growth of cultured meristems is about 2.5 times greater on Difco Purified agar than on Difco Special Agar, Noble. Other agars give intermediate results. The point to be emphasized is that agar cannot safely be regarded as an inert substance in a culture system. Before valid conclusions can be drawn concerning the effects of added supplements or regulators to the medium, it must be ascertained that growth is not being determined or limited by the agar. This consideration led to a study of a second kind of agar effect, that which results from changing the agar concentration.

AGAR CONCENTRATION

The selection of a type of agar which is superior to others, in terms of growth response, at a 1-percent concentration, does not eliminate the possibility that growth is still limited by some characteristics of the agar.

Growth of meristems is approximately doubled when concentration of Difco Purified Agar is reduced from 1 to 0.125 percent. The latter value is close to the lower limit at which the agar will form a gel firm enough to support a culture and to withstand careful handling of the dishes. Details of these experiments will be published separately. A brief statement of their significance appears below:

A plot of meristem growth against the logarithm of agar concentration is almost a straight line. Growth increases as agar concentration decreases. This is what one would expect if the diffusion rate of large molecules through the agar gel were a growth limiting factor. A low diffusion rate toward or away from a meristem culture (along radius r) would decrease the effective volume ($\pi r^2 h$—with h being small and near constant) of medium from which substances essential to growth of the culture could be drawn. Such a low diffusion rate would likewise decrease the effective "sink" volume into which inhibitory "exsorbates" (Heller 1965) could be diluted. The applicability of the effective volume concept is further suggested by observations of the volume into which a water soluble dye diffuses in a given time after application to the center of plates of gels of different agar concentrations. Graphing this volume as a function of agar concentration results in a curve very similar to that representing the effect of agar concentration on the growth of meristems. Present data do not allow a decision between local accumulation of exsorbates or local exhaustion of essential substances as the usual growth limiting condition in culture. Experiments on this point are underway.

It is also relevant to point out that the meristem cultures here discussed are very small—seldom exceeding 500 μg. fresh weight even

after 3 weeks of rapid growth. In the much larger callus cultures frequently discussed in the literature, internal diffusion may already be so limiting that agar concentration effects of the type we observed would not be evident.

DATA REPRODUCIBILITY PROBLEM

THE SITUATION

The difficulties of obtaining quantitative growth data from individual cultures are formidable. Even with a structure as large as a whole embryonic shoot (typically 2- to 8-mg. fresh weight), it is not easy to provide sufficient protection against desiccation to insure a reliable fresh weight and also to keep the tissue sterile during handling. Furthermore, there is no way in which one can accurately determine the dry weight of an excised embryonic shoot about to be committed to culture and still not jeopardize its survival. In contrast, embryonic shoots are large enough so that dimensions can be compared visually and any desired selections can be made under a dissecting microscope at low magnification. Such simple comparisons before and after culture treatments allow detection of qualitative and gross quantitative differences in responses. If more precise quantification is necessary, a modification of the procedure described below for apical domes can be adopted.

The problems posed by excised apical domes (typically 200μ tall, 250μ in diameter, and having a fresh weight of 10 to 20 μg.) are such that the only feasible approach to quantifying growth data is that of working with "matched" sets of meristems rather than with individuals, as such. Of the several matched sets prepared for an experiment begun on a specific day, one is sacrificed initially to provide a fresh-weight and dry-weight base from which final mean growth factors (final weight divided by initial weight) can be calculated for each of the other sets in that experiment. Matched sets to be used in a particular experiment are all composed of apical domes selected from those isolated during the same 6-hour period from plants having the same cultural history. This task is done as follows:

SET MAKEUP

Apical domes are excised, as previously described, from leading shoot tips of actively growing seedlings selected for normal vigor and growth habit. As nearly as possible, all domes are excised at the same morphological level—immediately above the youngest visible primordia—even though the physical heights of the domes vary considerably. As they are isolated, the apical domes are set in a row along one side of a 35-mm. dish of agar medium upon which nine disks (3 mm. in diameter) of cellulose polyacetate (CPA) filter membrane (5μ pore size, 140μ thick) have previously been laid out in a 3 by 3 pattern (fig. 8).[6]

After at least 14 domes have been isolated and after at least 15 minutes have elapsed since the last dome was placed on the agar (to allow for dilution and diffusion away of "wound" substances released by cutting of cells at the excision plane), nine are selected visually (at 30X stereo-magnification) on the basis of uniformity of size, shape, and general appearance. Each of these nine is then transferred to the center of a CPA disk and allowed to settled for at least

15 minutes before height measurement. Each CPA disk and the meristem it bears is assigned a number (1 to 9 on the first "9-spot plate," 10 to 18 on the second, etc.). Two experienced operators can prepare six such sets of nine meristems in 5 to 6 hours.

Height measurements of individual meristems are made optically with a compound microscope having a 10X objective, 15X eyepieces, a line adjustment barrel calibrated in microns, and a black opaque slide on the stage. Oblique surface illumination is provided by a focusable microscope lamp with a heat-absorbing filter. A transparent plastic dust shield fitted around the objective helps keep airborne contaminants from falling into the open plate containing the meristems.

The microscope is first focused on the surface of the CPA disk, and the barrel of the fine adjustment knob is read to the nearest micron. A similar reading is taken after focusing on the highest feature of the dome. The height in microns is computed from the difference between readings. At least three pairs of readings are taken from each meristem. The indicated mean height thus determined is usually repeatable to within 2μ to 3μ. Larger deviations generally indicate mechanical problems, unsuitable lighting, or excessive evaporation during measuring.

After all meristems to be included in 1 day's experiment are measured, they are ranked according to increasing height. Nine are measured and ranked for every seven to be selected for culture. Thus, about 22 percent of the meristems near the extremes of height can be rejected. Remaining individual meristems are then assigned to sets in such a way that the summation of heights of individuals in each set (typically $1{,}400\mu$ to $1{,}500\mu$) is within $\pm 2\mu$ of the mean of such values for all sets and so that the distribution of heights within each set is comparable. Random selection methods are used to designate one set to be sacrificed for initial weight determinations and to assign the others to particular treatments. The prepared and numbered dishes of experimental culture medium (seven dishes for each set) are then laid out, and the appropriate transfers of CPA disks and meristems are made with a sterile microfork. The sets are then ready for incubation.

Sets prepared in this way are, of course, not identical. In one experiment four such "matched" sets were all sacrificed on the day of excision. Dry weights per set ranged from 16.7 to 18.7 μg. (total dispersion 11.2 percent of the mean). Hence, errors

[6] The cellulose polyacetate (CPA) disks serve several purposes. They provide a slightly textured base from which to measure the height of domes. They make possible the transfer of cultures from dish to dish without necessitating any tool-tissue contact, and without the aid of a microscope. Thus, cultures can easily be removed from agar plates which show microbial contaminants not yet contacting the disk.

These disks are cut from larger filter disks with a paper punch. Leachables (mainly glycerol) are removed by boiling for 30 minutes in each of three successive changes of distilled water. The wet, leached disks are laid out, with no contact or overlapping, on the bottom of a Petri dish and autoclaved (slow exhaust). Best results are obtained if sterile disks are transferred to agar plates while still slightly moist from autoclaving. If the disks are allowed to become thoroughly dry, subsequent rehydration is very slow. Leftover disks should not be resterilized for later use. Repeated autoclaving has deleterious effects upon CPA membrane.

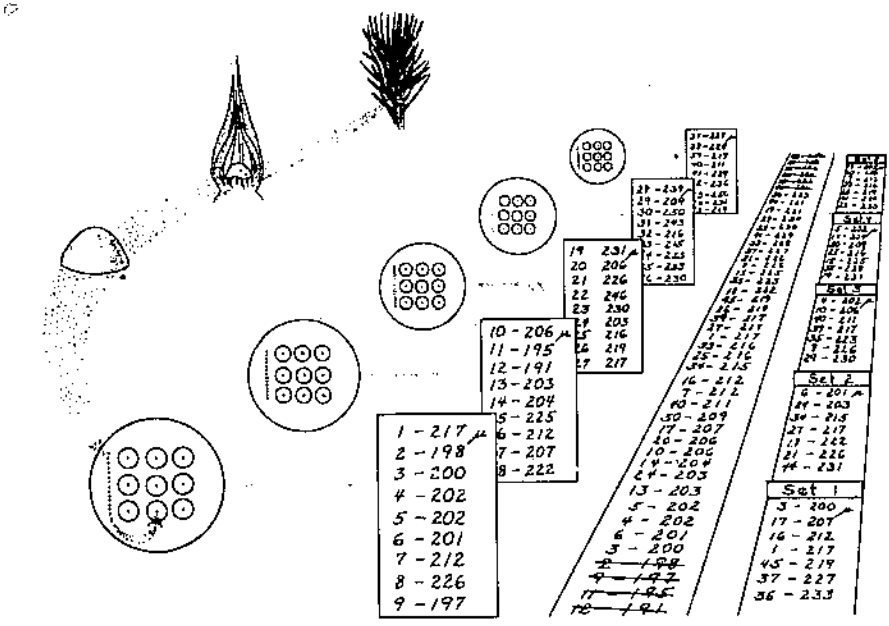

Figure 8.—A schematic representation of the steps involved in preparing "matched" sets of *Picea abies* meristems for experimental culture. The steps are: Removal of leaves and larger primordia from the shoot tip to expose the apical meristem on one side, excision of the apical dome, transfer of the dome to an agar plate (14 domes per plate), selection of nine of the domes for uniformity of appearance and transfer of them to cellulose polyacetate disks, measuring height of the domes on each "9-spot plate," ranking all the domes according to height, rejecting those having extreme values, and grouping the remaining domes into matched sets.

as large as 10 to 12 percent could arise from applying the weight of the sacrificed set as an initial weight of all "matched" sets prepared on that day. However, final dry weight differences between treatments are typically much larger than this, and we have also adopted the practice of repeating each test at least five times before attempting to assign significance to the results.[7]

[7] Indeed, the limitations imposed by the material are such that the only way to evaluate the statistical significance of treatment differences is to repeat each test several times. It is not feasible to obtain a measure of within-treatment variance on the basis of dry weights of meristems within a single set because individual weights at the microgram level can be obtained only at great time cost and would have increased variability. Hence, the whole-set dry weights of each of the several sets cultured as replicates of the same treatment are used to calculate within-treatment variance. All sets subjected to each treatment are then grouped together for calculation of between-treatment variance. Meristems in sets in the same test are matched as described above, but, because they are prepared on different days, no attempt is made to match meristems between treatment replicates. They are similar, but mean heights and weights do vary somewhat. Thus, another source of variance—that between sets not matched and derived from plants of similar but nonidentical histories—is introduced and is not accounted for. The effect of this is to increase within-treatment variance and to reduce the likelihood of detecting small but "real" differences between treatments. The inability to detect small differences, however, is not a serious deficiency. If between-treatment differences are not large and fairly obvious, they are of little value in elucidating the physiological systems by which growth and development are controlled.

OBSERVATIONS AND MEASUREMENTS OF GROWTH

Increase in weight is an easily quantified measure of growth, but it would be a great mistake to ignore entirely the other aspects of development and morphogenesis merely because they are difficult to reduce to numerical data. The weight measurements discussed in the preceding section may completely fail to reveal developmental or morphological differences obvious to the eye. We feel it desirable to record such differences, even though we cannot yet explain or interpret them. So little is known about the behavior of apical meristems in culture that even the design of good experiments is inhibited. In making periodic growth observations, our primary objective is to learn what to expect from meristems growing under various conditions. The use of this "experience information" in the design of experiments will come later, but it is pointless to attempt to predict now just how this will occur.

Ordinarily each individual culture is observed at 45X magnification once each week. Notes are made on the color, surface characteristics, general vigor, and on number, arrangement, and shape of any primordia present. Although observations such as these are not readily reduced to numerical data, they allow the developmental history of an individual meristem of a known initial height to be reviewed at any time.

Even so simple a concept as number of primordia does not lend itself to ready quantification because the isolated meristem does not always confine its activities to the initiation of discrete primordia (as

Figure 9.—A 3-week-old meristem culture which has developed large discrete primordia above a short basal stalk. Such cultures usually undergo a 20- to 30-fold dry weight increase in 3 weeks. (64X)

in fig. 9). The actual pattern of development is partly determined by the height above the youngest primordia at which the apical dome is excised. There may be primordial ridges, buttresses, or annuli of all descriptions (e.g., see fig. 10). These

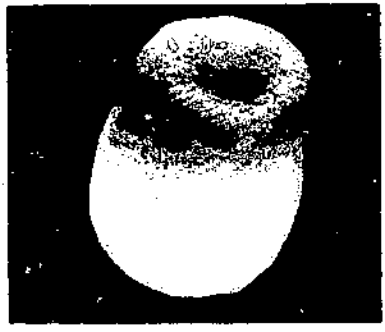

Figure 10.—A cultured meristem developing a primordial annulus rather than discrete primordia. Such an annular ridge may grow upward as a tube for a short distance, and then become segmented into lobes with multiple tips. (64X)

may or may not develop into distinctly separate lobes or primordial leaves as they grow. In addition, the very large, broad, and vigorous first-formed primordia (sometimes with multiple tips) often cup over the apex and hide the younger, more acute-tipped, more nearly "normal" primordia that may be initiated later. This behavior, as an example, illustrates why attempted quantification of a variable such as number of primordia is not yet likely to be rewarding.

It is sometimes valuable and instructive to measure the growth in height of meristems in culture. The measuring technique used is the same as that described earlier. During the first 10 days of height growth, values of 20μ to 40μ per day may be expected. But growth in height is not necessarily correlated with overall vigor or increase in weight. Some cultured meristems become tall and slender with a long stalk below the lowest primordia (fig. 11), whereas others with similar weights are short and stocky with numerous heavy primordia surrounding the dome (fig. 9). Developing primordia will usually cover and hide the dome during the second or third week of growth, and meaningful height-growth measurements then become impossible. Height measurements have value primarily when used to graph early trends.

Figure 11.—This 3-week-old meristem culture produced a long "stalk" before primordia developed. (70X)

WEIGHING

The weight determinations mentioned earlier require an electro- or other micro-analytical balance having a sensitivity of at least 0.1 μg. for sample weights of less than 1 mg. Disposable dish-shaped aluminum foil weighing pans (12 mm. in diameter) are used for all weighings. Loading of sample pans must, of course, be done under the microscope, and safeguards must always be taken against the possible loss of samples or contamination by dust.

Estimates of fresh weights of sets of cultures or meristems are obtained as follows: An accurately counterbalanced sample pan is put inside a 2-inch weighing dish (as a catch basin) on the stage of a dissecting microscope. Approximately 5 μl. of distilled water is put on the 12-mm. sample pan. The water forms a high-domed bead. Individual cultures are transferred with

a slender microscalpel from the CPA disks on which they were growing to the water bead. When the meristem or cultured organ touches the water surface, it will usually suddenly "jump" from the scalpel to the lower margin of the bead and will next be seen (through the microscope) in profile there. After all cultures or meristems have been so placed and counted, the water is withdrawn by use of a microcapillary. This must be done quickly and completely, but usually requires some gleaning of the last microscopically visible free water with a second, smaller capillary. The pan is then quickly transferred to the instrument stirrup, and a balance is quickly obtained (within 5 sec. or less). The cultures must be removed from the pan within a few minutes; otherwise they tend to stick. They are placed in a glass microcup for drying.

Drying of apical meristems and cultures derived from them for 2 hours at 82° C. has been adequate in our experiments for reliable dry-weight determination. The samples are allowed to cool in the presence of a silica gel desiccant. Transferring the dry, brittle pieces to a weighing pan must be done with great care because any crinkling or snapping of the foil weighing pans may catapult some of the tissue masses out of the field of the microscope.

Obtaining dry weights of embryonic shoots (several hundred times larger than apical meristems), particularly in the winter when sugar concentration is high, requires very long drying periods. The problems are the same as those involved in drying syrups. Even lyophylization followed by 24 hours in vacuo over phosphorus pentoxide does not result in drying to constant weight. Several weeks of drying under the latter conditions approaches adequacy.

PHOTOGRAPHY

Often the only adequate description of a developmental sequence or of a particular structure is a set of photographs; this is especially true if the objects being studied are very small. Yet the usual methods of photomicrography are too "micro" and ordinary closeup photography is too "macro" for objects such as meristems and their cultures. Whole embryonic shoots, however, present no optical problems which cannot be handled with a 35-mm. camera with a through-the-lens viewfinder screen, extension bellows, and a reversible lens. Depth of focus, lighting, and desiccation are, of course, perennial problems with which one must reach a compromise in each situation. Agar gel is a suitable background for such photography if vibrations are damped out and exposures are not excessively long. Two focusable microscope lamps (with heat filters) and a polarizing filter are very useful in arranging the lighting. Beyond that, experience with the particular system available is the only guide.

We approached the problem of photographing the semitransparent, near microscopic, apical meristems by using low-power objectives (2X, 3.5X, and 5X) in combination with high-power oculars. In doing so, of course, one sacrifices magnifying power and numerical aperture of the objective lens to gain working distance and depth of focus. There is an unavoidable loss of resolving power which a high-power ocular cannot restore. In this situation only compromise is possible. Never-

theless, moderate persistence and attention to detail will allow photography of living meristems and cultures at 20X to 100X magnification without damage or contamination.

Lighting is best provided at a low angle above the horizontal from two sides about 180° apart. The technique is to make the object appear to glow from internally reflected and diffused light. A polarizing filter, replacing the lid of the Petri dish, is rotated until surface reflections are minimized. Thus, cultures can be made to appear in realistic tones (depending upon cell size and degree of vacuolation) against a background which is completely black in the final prints. Illumination by this method is never brilliant; hence, high-speed film and moderately long exposure times are necessary. Overdevelopment and contrast-promoting printing techniques may also be required. Any vibration or any movement due to desiccation of the agar will, of course, cause blurring.

GENERALIZATIONS—TECHNIQUES AND RESPONSES

If the procedures described herein are followed, more than 95 percent of all explanted apical domes should survive for at least 1 week and exhibit some growth. Incidence of tissue-borne microbial contamination should be less than one in 500. Although meristems sometimes slowly decline and die of unknown causes in culture, such losses are also often traceable to initial damage or injury. Fortunately it is usually possible to recognize the early symptoms of such damage and to avoid selecting damaged individuals for inclusion in experimental sets.

Excessive mechanical disturbance and distortion of the cellular structure of the meristem during excision often results in gelation of the protoplasmic sol. This damage can result from poor design, dullness, or inept use of microtools. A meristem so damaged may initially retain its normal shape and turgor, but the damaged cells appear dense whitish-translucent in distinct contrast to the clear, almost transparent cells more distant from the cut (fig. 12). Whereas meristems with such whitish basal areas may recover, those areas may also turn brown and presage the demise of the whole structure. Likewise, any cut (or microscopically visible bruise or abrasion) anywhere on the surface of the dome should be disqualifying. If the cut is clean, with distinct margins, the meristem may survive, but it probably will be distorted and asymmetrical.

Aside from the above considerations, practically all meristems will survive and grow. Whereas little pigmentation may be evident initially, 1-week-old cultures (when grown in the light) are pale green, and the green deepens with time. Furthermore, those primordia initiated later are darker green than those iniated earlier. If the initial excision is made so that the explant includes the youngest primordia surrounding the apical dome, these almost never grow and develop. Instead, the dome grows rapidly upward during the first week. New primordia are then initiated, leaving a "stalk" below. To assure a degree of comparability between meristems isolated at various times from different crops of seedlings, we generally excise at that level which will include all of the dome but no primordia (fig. 13). Such domes will generally have initiated

Figure 12.—This meristem, photographed just after excision, shows evidence of extensive damage due to dull tools or inept handling. The cells of the whitish areas may not survive. (131X)

Figure 13.—A freshly isolated apical dome having no primordia or visible sites of initiation of primordia. Such domes are almost colorless and quite translucent. (64X)

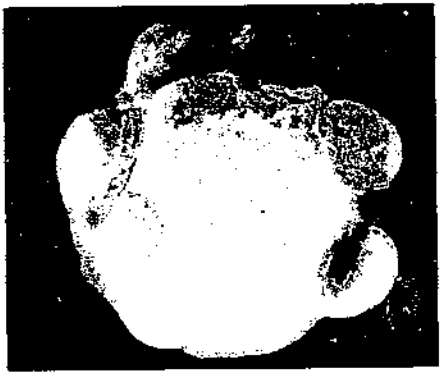

Figure 14.—After 1 week in culture, a meristem initially similar to the one shown in figure 13 has initiated at least 10 visible primordia and has become light green. (80X)

four to 10 new primordia by the end of a week (fig. 14), but their number, shape, and arrangement is highly variable.

It is entirely proper to ask whether the growth and development of meristems here illustrated is merely due to water uptake or whether cell division and dry-weight increase also occurs. These questions are easily answered. One-week-old cultures have been killed, fixed, and stained. Division figures definitely are present. With suitable lighting and optics, dividing cells can also be seen in the apical regions of living, fast-growing primordia and in the living apical dome itself. Dry-weight increases of 20- to 30-fold in a 3-week culture period are routine, but the substances which are synthesized have not yet been identified.

It must not be assumed that 3 weeks represents a limit beyond which the cultures will not survive. Growth can continue for 6 weeks or more on the basal medium (fig. 15). Three weeks is merely a convenient compromise between various problems of time, space, and resources. We have kept cultures alive for more than 8 months, but have not yet made concerted efforts to isolate substances or to define conditions necessary for long-term survival and growth. Short-term experiments involving various physical parameters and addition of supplements to a well-defined, near minimal, culture medium seem more relevant at this stage of the work.

Many types of experiments not mentioned in the foregoing are facilitated by these techniques. Meristems can be isolated from all types of growing shoots or dormant buds. They can be grafted together, or they can be transferred from one shoot to another. With care they

can be halved and the pieces can survive separately or recombined. The apical dome can even sometimes be excised afresh from 3-week-old cultures and can be subjected to additional culture on a different medium.

We have not done extensive work with species other than *Picea abies*, but that which we have done suggests that the techniques themselves are applicable to other conifers. Growth responses have varied with species, but, in general, those of *P. abies* have been more satisfactory than any other.

Finally, it is emphasized that apical meristem culture is not *tissue* culture but *organ* culture. Growth and development is organized and much closer to that of the intact plant than that of calus masses. Of the more than 4,000 *Picea abies* meristems we have cultured, none ever produced callus tissue. Organized growth is initially encouragingly rapid, even on a simple medium. The ultimate in rapid and sustained growth certainly has not yet been reached.

Figure 15.—After 6 weeks on basal medium, this culture looks green and vigorous, but it is making little additional externally visible growth or development. Such cultures show no tendency to form basal callus. (32X)

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