CLONAL PROPAGATION OF *ACACIA KOA* GRAY BY TISSUE CULTURE AND CONVENTIONAL METHODS

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ABSTRACT

Asexual propagation techniques were required to vegetatively increase large *Acacia koa* Gray trees that had been selected for their superior growth and form in the forest. Experiments indicated that only material in the juvenile condition was suited to this purpose. Root suckers and stem sprouts were the source of material from the mature, forest-grown trees. Material from seedlings was also used in some tissue culture work. Success was achieved with tissue culture of seedling material and unrooted shoots were obtained from root sucker material. Root suckers were also successfully propagated by mist rooting of cuttings, and air layering, but not by other methods which included grafting, aseptic organ culture, and shoot induction from root cuttings.

Numerous complete plants of one clone and shoots of another were produced by aseptic culture of shoot tip tissue. Shoots were formed in cell suspension cultures as well as from callus on agar. Callus was induced and increased most effectively in a liquid basal medium of Murashige and Skoog supplemented with 1.0 mg/ℓ each of 2,4-dichlorophenoxyacetic acid, napthaleneacetic acid, para-chlorophenoxyacetic acid, indoleacetic acid, benzyladenine, 6γγ dimethylallyladenine, zeatin, kinetin, and benzylaminobenzimidazole.

Callus from a seedling shoot tip formed shoots during sequential benzyladenine, coconut milk treatments. Shoots of one seedling shoot tip callus proliferated and elongated on a low benzyladenine medium and were rooted in agar media containing indolebutyric acid. Functional roots were obtained by growth in a liquid medium. Plants

required intensive aftercare before planting in the field, where they developed into trees of normal appearance.

Callus from root sucker tips formed shoots in suspension cultures and on agar media containing dimethylallyladenine. Shoots were elongated on the basal medium without growth regulators and occasionally formed non-functional roots on low salt media or on media with reduced sucrose.

The three successful methods--tissue culture, air layering, and mist rooting--are as yet practical only for experimental purposes. All need more refinement if they are to be used for mass propagation. Clonal variation among propagules may be large. The practicality of vegetatively propagating *Acacia koa* must be determined by clonal progeny tests of the plants being produced. Propagules in one such test have shown possible problems with topophysis.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (Continued)

LIST OF ILLUSTRATIONS (Continued)

LIST OF ILLUSTRATIONS (Continued)

LIST OF ABBREVIATIONS

Adn - adenine

B - N^6 benzyladenine

Ben - benzylaminobenzimidazole

CM - Coconut milk (water)

C - para-chlorophenoxyacetic acid

D - $N^6\gamma\gamma$ dimethylallyladenine or 2-isopentenylallyladenine

GA - gibberellic acid

BT - benzthiazolyloxyacetic acid

IAA - indole-3-acetic acid

IBA - indole-3-butyric acid

K - kinetin, N^6-furfurylamino purine

NAA - napthaleneacetic acid

Z - zeatin, N^6-4-hydroxy-3-methylbutyl-2-enyl amino purine

2,4-D - 2,4-dichlorophenoxyacetic acid

General Introduction

Koa (*Acacia koa* Gray) is Hawaii's only native timber tree that has valuable wood. *Acacia koa* is an endemic Hawaiian phyllodinous Leguminosae of the subfamily Mimosoideae. It is a tree which occurs on all the principal islands of the Hawaiian chain, achieving its largest size between 1200 and 1700 meters elevation on the slopes of Mauna Loa, Mauna Kea, and Hualalai on the island of Hawaii. It achieves a height of over 30 meters and a breast height stem diameter of as much as 3 meters at its maximum development.

Much of the former koa forest has been converted to grassland and much of the remaining forest contains numerous feral pigs and cattle which destroy young koa trees. In addition, many of the largest, straightest trees in the forests have probably been removed during a period of several centuries and used for the manufacture of dugout canoes. Consequently, trees of a size and form best suited for the manufacture of lumber and veneer are becoming rare. If such trees are to become again an important component of the forest, the few remaining very straight, well-formed trees will have to be increased by propagation.

To this end, 46 of the best remaining trees on the islands of Hawaii and Kauai were selected for their straightness, size, and branch-free stem characteristics to be used for propagation. Almost no vegetative propagation techniques had been reported for koa so it was necessary to determine if vegetative propagation could be used to produce identical progeny or clones of the selected trees.

The objective of the studies reported here was to determine if

large, mature koa trees could be reproduced asexually by one or more techniques of vegetative propagation. The techniques were to be used to clone large trees of selected characteristics.

Numerous vegetative propagation techniques were applied to koa. They included the callus and cell suspension methods of tissue culture, as well as the more conventional techniques of grafting, air layering, and mist rooting. Because most of the experimentation was concerned with tissue culture, three of the following four chapters deal with that subject. The fourth chapter presents the studies of conventional propagation techniques.

General Literature Review

Probably because koa is easily propagated from seed, very little vegetative propagation work has been done with it. Baldwin and Fagerlund (1943) were the first to examine and report its ability to regenerate by root suckering, which they attributed to a reaction of the tree to cattle damage. Lanner (1965) also recognized root suckering in the same area, the Mauna Loa strip. According to Whitesell (1964), Lanner also observed natural layering. Mueller-Dombois (1967) and Spatz and Mueller-Dombois (1973) suggested that root sprouting is caused by damage by feral goats. Spatz (1973) further observed that the solar heating resulting from grass removal by grazing might cause suckering.

Callus was produced from a succulent mature (phyllode) shoot tip obtained in Manoa Valley in 1970 (M. Mapes, laboratory records). The callus was grown for 2 1/2 years in liquid media and produced a cell cluster suspension which formed embryoids. Shoots, however, failed to

develop and the culture was eventually discarded.

A rooted plantlet was grown from koa seedling shoot tip callus in 1975 (Skolmen and Mapes 1976). A search of the literature has indicated that this was the first success reported of tissue culture propagation of an *Acacia*. Three other references concerned with tissue culture of acacias were found.

Bonner (1942), using White's medium (White 1939) in liquid form, grew a root of *Acacia melanoxylon* R. Br. continuously for over 1 year. The root exhibited neither diameter enlargement nor lateral roots. McLean et al. (1966) grew callus from *Acacia harpophylla* F.v.M. seedling stems using White's medium (White 1963) with 2,4-dichlorophenoxyacetic acid (2,4-D) and 15 percent coconut milk. Winton (1974) reports corresponding with Zeijlemaker who grew callus of *Acacia mearnsii* De Wild. from cambial tissue, but discarded it when it was found to have retained a wood-pitting virus that the researcher had hoped to eliminate. The third reference was Kathju and Tewari (1973), which is cited by Pierik (1975) as concerned with tissue culture of acacias.

The lack of successful tissue culture propagation, or complete regeneration of plants of *Acacia* is not unique to the genus. The difficulty of tissue culture propagation of all woody plants has been noted by several workers in the field. For example, Pierik (1975) states: "Vegetative propagation of herbaceous plant species in vitro appears to be relatively easy, but vegetative propagation of trees and shrubs (angiosperms and gymnosperms) in vitro is very difficult. The overall conclusion is that in woody plants the capacity (ability) to

regenerate is much lower in comparison to herbaceous plants."

Murashige (1974b), in reviewing plant propagation through tissue culture said, "---the conclusions of this review should not be viewed as applicable to the propagation of woody perennials, except as a foundation for their research." The conclusions of the reviewer were based on results obtained with herbaceous plants.

Pierik (1975) listed 74 non-coniferous shrubs and trees of 41 genera as having been regenerated in vitro. However, he did not differentiate between those produced by de-differentiation and re-differentiation of secondary tissue and those produced by embryo culture, nucellus culture, aseptic grafting and shoot tip rooting. These other methods are not considered very significant by many workers in the tissue culture field. Winton and Huhtinen (1976) listed 18 species, varieties and hybrids of angiosperms, and one palm that have produced rooted shoots from callus culture. Eight genera are represented among the 18 species; 11 *Populus* spp. and two *Citrus* spp. were on the list. Durzan and Campbell (1974) listed 34 genera of broad-leafed forest trees for which propagation by tissue culture was attempted. Among these only four species of forest trees produced plantlets. These were three species of *Populus* and *Eucalyptus citriodora* Hook. Plantlets have been produced by tissue culture of quite a few conifers, several citrus, almond, and oil palm, but only a few angiospermaceous forest trees have yet been propagated by tissue culture.

CHAPTER 1

INDUCTION OF CALLUS FROM VARIOUS TISSUES OF *ACACIA KOA*

Introduction

The mass propagation of *Acacia koa* by callus or cell suspension culture required that callus be induced to form from plant parts or tissues retaining the potential to regenerate into complete new plants, in other terms, tissue retaining totipotency. It also required that the media used to induce and grow callus and cell suspensions support rather than inhibit the totipotency of the cultures.

It was first necessary to find which plant parts or tissues could be made to de-differentiate or form callus under the influence of various basal media and growth regulators. Later, it was determined which cultures retained the capacity to regenerate organized tissue and whole plants.

Literature Review

It is well documented that among woody plants, juvenility of tissue is important to success in vegetative propagation (Hartmann and Kester 1975). This has proved to be true in tissue culture propagation as it is with conventional propagation (Murashige 1974b, Pierik 1975, Winton and Huhtinen 1976). With the possible exception of *Sequoia sempervirens* Endl., from which rooted plants have been grown (Ball 1950), the literature does not indicate any forest tree that has been propagated from callus or cell suspension cultures induced from mature tissue. The Sequoia plants were produced from callus derived from burl tissue, which in nature forms juvenile adventitious shoots.

Aneja and Atal (1969) produced *E. citriodora* Hook. plantlets from callus produced from a lignotuber which is a special structure formed at the root crown of some eucalypts which retains the capacity to sprout if the stem is cut back or dies. Staritsky (1970) produced plantlets of *Coffea canephora* Pierre ex Froehner, from callus formed from soft green stem sections of seedlings. Winton (1970) used stem segments of very young greenhouse-grown root sprouts to obtain callus from which he obtained aspen (*Populus tremuloides* Michx.) trees. He (Winton 1971) used similar material to produce trees of European aspen (*P. tremula* L.) as well. Mehra and Mehra (1974) obtained whole plants of *Prunus amygdalus* (Tourn.) L. from callus of 7- to 8-day-old seedling cotyledons and leaves and from callused embryos.

Populus and *Ulmus* are the only trees so far produced from tissue that had been in cell suspension. *Ulmus americana* L. was started from 10-day-old seedling hypocotyl segments (Durzan and Lopushanski 1975). *Populus tremula* seedling stem cells were shaken from callus in liquid, then plated out and grown into callus again, and then into whole plants (Rion et al. 1975). Herman and Haas (1975) obtained numerous whole plants of *Coffea arabica* L. from callus obtained from leaves of some greenhouse-grown plants.

There are other examples, but these indicate that the ability to differentiate is retained in tissue from a number of plant parts so long as those parts are young.

In general, with the notable exceptions of carrot and bindweed tissue, it has been found that callus obtained from root tissue will regenerate only roots (White 1963, Murashige 1974b). Tissue from

seedling tips and hypocotyls is considered more likely to demonstrate totipotency than that from stems. Though leaf tissue callus is capable of regenerating plants (Herman and Haas 1975), it does so less frequently than other tissues. This indicates that in addition to juvenility, the location of the tissue within the juvenile plant is important to totipotency of callus derived from it.

The potential to form callus varies with the ontogenetic stage and location of the tissue in the plant (Murashige 1974b, Yeoman 1973). Although most woody-plant tissues will form callus, if in a succulent stage when explanted, they show variation in rate of reaction to various media (Pierik 1975).

Numerous basal media and growth regulators have been used to obtain totipotent callus from forest tree tissues. The basal medium that has been used most frequently is that of Murashige and Skoog (1962), or modifications of it such as those of Linsmaier and Skoog (1965) and Brown and Lawrence (1968). Murashige and Skoog's medium was developed for growing tobacco callus, but is excellent for many other tissues (Gamborg et al. 1976). Another basal medium now widely used for tree callus culture is that of Schenk and Hildebrandt (1972), which was originally developed to support callus growth of numerous herbaceous genera. These media contain mineral nutrients, vitamins, and other substances required for plant growth. The formulation of Murashige and Skoog's and Schenk and Hildebrandt's media are given in Appendix A.

As well as sucrose, which is used as a carbon source (Gamborg and Wetter 1975), and vitamins, various growth regulators are added to

basal media to promote de-differentiation of tissue, cell division, and differentiation of callus (Yeoman 1973). Tissue culturists have experimented with many different auxins and cytokinins to control callus formation and growth (Yeoman 1973). Usually, the auxins and cytokinins have been added singly, that is, one auxin and one cytokinin at a time. Some researchers have combined them. Mapes and Urata (1970) combined adenine and coconut milk, two substances that cause cytokinin-like reactions in plant tissue cultures. Schenk and Hildebrandt (1972) combined a small amount of 2,4-D with a larger amount of para chlorophenoxyacetic acid to obtain a strong callus induction-cell enlargement auxin effect without causing the aberrations or ploidy problems associated with high levels of 2,4-D (Murashige 1974b). Cheng (1975) reported using mixed auxins and cytokinins to obtain buds on Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] seedling cotyledons. Kakade and O'Connor (1977) used mixtures of two auxins and two cytokinins in embryo culture of Douglas-fir.

The auxins, which are used in plant tissue culture for their effects in causing de-differentiation and cell enlargement (Gamborg and Wetter 1975) are known to stimulate RNA synthesis (Key 1964, Stewart, Mapes, Kent and Holsten 1964, Yeoman and Mitchell 1970). They also stimulate cell wall plasticization (Heyn 1931, Bonner 1960). They are thought to be activated by attachment to some stereospecific site (Thimann 1963) because all the important auxins have the same structural distance between their positive and negative poles. However, they are highly variable in their effects on various tissues,

being most effective on stem and coleoptile tissue (Leopold and Kreidemann 1974, p. 123). Tissue culturists who have used several auxins on the same types of tissues (Mehra and Mehra 1974, White 1963) report variations in their effectiveness.

Cytokinins are also known to increase RNA and in fact the natural cytokinin 6γγ dimethylallyladenine, has been found to be a part of certain tRNA as has zeatin for other tRNA (Hall et al. 1967). Fox and Chen (1967) were able to implant radioactive benzyladenine into the RNA of soybean seedlings. However, it is uncertain that their presence causes a direct influence on protein synthesis (Leopold and Kreidemann 1974). There is some evidence that cytokinins increase auxin content (Skoog and Armstrong 1970), as well as gibberellins (Loveys and Wareing 1971), and ethylene (Fuchs and Lieberman 1968). Benzyladenine has been found to form a stable compound, 6 benzylamino-7-glucofuranosylpurine when added to cultures of various plant tissues (Deleuze et al. 1972). Parker et al. (1972) found a similar stable derivative for zeatin, which they called raphanatin. These long-lived materials may be incorporated in the cytoplasm and play long-lasting rolls even after transfer of the tissues to other media.

The activity of cytokinins varies as does the activity of auxins (Leopold and Kreidemann 1974, p. 158). With one plant (*Xanthium*), activity (cotyledon enlargement) increased in relation to concentration with all cytokinins. Benzyladenine was much more reactive than the others tried, which fell in the order zeatin, tetrahydropyranylbenzyladenine, 6γγ dimethylallyladenine, kinetin,

diphenylurea, and adenine.

These findings with auxins and cytokinins suggest that used in combination, they might supply augmentive, though independent activities. They might also supply required molecular rings, acid side chains, or be able to occupy several different stereospecific sites that would not be supplied or occupied by only one auxin and one cytokinin (Thimann 1963, Leopold and Kreidemann 1974, p. 125-127). Such factors might result in increased activity in the cultured tissue.

Materials and Methods

All tissue culture experimentation was carried out at constant temperatures of 25° C in one or the other of two laboratories. Continuous light was used for most experiments, but intensity and qualities used varied somewhat between culture methods and laboratories. In one laboratory, callus, shoots, and rooted shoots on agar slants were grown at a photon flux density (photosynthetically active radiation) of 46 to 62 micro einsteins per square meter per second ($\mu E\ m^{-2}sec^{-1}$). Liquid cultures of callus and cell suspensions were grown at approximately 16 $\mu E\ m^{-2}sec^{-1}$ on a gyro-rotary shaker at 60 rpm. In the other laboratory agar slants were kept at 30 to 66 $\mu E\ m^{-2}sec^{-1}$ and liquid cultures were shaken at 100 rpm under a photon flux density of 16 to 24 $\mu E\ m^{-2}sec^{-1}$.

Plant material used included embryos, aseptically germinated seedlings, field-grown seedlings, root suckers, adventitious shoots, root cuttings, and cuttings from mature trees. Field-collected material was rinsed in 70 percent ethanol to kill mites. Explants

were prepared from this material by removing the desired portion and surface sterilizing it in a 15 percent v/v solution of laundry bleach (5.25 percent sodium hypochlorite) in water to which a drop of detergent had been added as a surfactant. After sterilization, the explant was rinsed in sterile water and inoculated to the culture medium under aseptic conditions.

Several basal nutrient media were tried for callus induction, growth, and differentiation. The principal one used was that of Murashige and Skoog (1962). Another common basal medium used was that of Schenk and Hildebrandt (1972). The formulations of these two media are given in Appendix A. Other basal media tried were White (1963), Knudson (1946), Vacin and Went (1949), and Nitch and Nitch (1969). The inorganic salts and vitamins of these media were used in the formulations published, but various modifications were made with some of the other organic constituents.

Organic constituents added to the basal media included auxins, cytokinins, gibberellic acid, and coconut milk. The auxins used were indole-3-acetic acid (IAA) parachlorophenoxyacetic acid (C), indole-3-butyric acid (IBA), napthalenacetic acid (NAA) and 2,4-D. The cytokinins used were adenine (Adn), N^6benzyladenine (B), $N^6{}_{\gamma\gamma}$ dimethylallyladenine (D), benzthiazolyloxyacetic acid (BT), benzylaminobenzimidazole (Ben), kinetin or N^6-furfurylamino purine (K), and zeatin or N^6-4-hydroxy-3 methylbutyl-2-enyl amino purine (Z). The coconut milk (CM) added was the liquid endosperm of green or unripened nuts. It was extracted and kept frozen until used.

These constituents were added to the basal nutrient media singly

or in various combinations at various concentrations as will be brought out in the results.

All media used were autoclaved at 121° C for 20 to 30 minutes. Agar, when used to gel the media, was at a concentration of 8 g/ℓ. The pH of the various media was set at the levels prescribed by the literature, except that the pH of Murashige and Skoog's medium was set at 6.0 before autoclaving rather than the recommended 5.7, because it was found that autoclaving reduced the pH to 5.7

Cultures were started and kept on agar slants containing 10 ml of media in 25 mm test tubes on lighted shelves, in 50 ml erlenmeyer flasks containing 15 ml of liquid media kept agitated on a gyro-rotary shaker, or in t-shaped tumble tubes containing 10 ml of liquid media rotated on a drum (Steward and Shantz 1956, Street 1973b p. 62). Explants showing slight contamination in culture were repeatedly surface sterilized and re-inoculated to new media until free of contamination. Severely contaminated explants were discarded.

Explants were from many different sources (Table 1) and required varied preparation methods. Embryos and cotyledons were excised aseptically from swollen seed that had been heat scarified by placing in water at 100° C which was then allowed to cool overnight. The seeds were surface sterilized before removing the seed coats.

Seedling tips, root sucker tips, and adventitious shoot tips consisted of the apical and axillary meristems with as many leaf primordia removed as was possible without destroying the meristem. All shoots were from material with true leaves. A typical root sucker tip as prepared as an explant is shown in microscopic section in

TABLE 1

INFLUENCE OF *ACACIA KOA* EXPLANT SOURCE ON SURFACE STERILIZATION SUCCESS, CALLUS INDUCTION, AND REGENERATION CAPACITY OF CALLUS FORMED

Source	Attempts	Not contaminated	Callus induced	Callus formed adventitious shoots
		Number		
Embryos	5	4	2	0
Cotyledons	18	16	0	0
Seedling				
tip	56	13	9	6
hypocotyl	25	4	2	1
stem	32	16	13	0
leaf	5	2	1	0
petiole	11	8	3	0
root crown	6	4	0	0
Root sucker				
tip	187	39	11	3
stem	78	27	14	0
leaf	13	6	2	0
petiole	41	14	9	0
phyllode	15	8	0	0
root sphaeroblast	32	4	1	1
Adventitious shoots				
shoot tip	37	9	2	1
shoot petiole	18	6	0	0
shoot stem	12	7	0	0
sphaeroblast	14	3	0	0
Mature tree				
phyllode shoot tip	93	11	1	0
tip of phyllode	4	2	0	0
phyllode tissue	7	4	0	0
flower buds, young	25	5	0	0
flower buds, mature	34	3	0	0
flowers	42	0	0	0
flower parts	28	8	0	0
peduncle	5	3	2	0
anthers	8	2	0	0

Figure 1. These were prepared under magnification by teasing free the outer leaf primordia.

Seedling hypocotyls were the curved portion of freshly germinated seedlings just below the cotyledons. Seedling stems were short lengths of that portion of the seedling between the hypocotyl and the root crown, which in koa seedlings, is a definite platelike structure.

Sphaeroblasts and root sphaeroblasts (Hartmann and Kester 1975 p. 240) are specialized globose protuberances that form on the stems and roots of koa trees from which arise adventitious shoots and root suckers. A root sphaeroblast (Fig. 2) when sectioned (Fig. 3) is seen to arise from the pericycle/endodermis region of old roots that have lost their cortex. Beneath the bark of the sphaeroblasts is a meristematic zone and it was this material that was used for sphaeroblast explants. These were prepared by removing the bark and cutting pieces of succulent green material from the sphaeroblast under a dissecting microscope.

Mature koa foliage consists of phyllodes and the shoot tips are of a very different structure than the true-leaf tips of seedlings and root suckers. Phyllode shoot tips were easily freed of all surrounding phyllode primordia. The tips of some phyllodes bear a pair of true-leaf rachises and it was these "tips of phyllodes" which were used as explants.

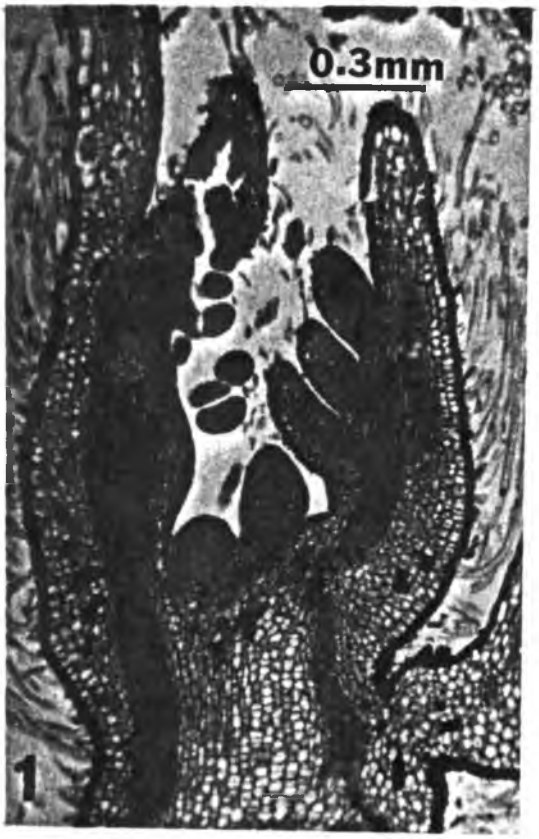
The flowers and flower parts used included 3 mm diameter tightly closed young inflorescences, 6 mm diameter well-developed inflorescences about to open, and also halves of these compact flower clusters. Also included were whole inflorescences, individual

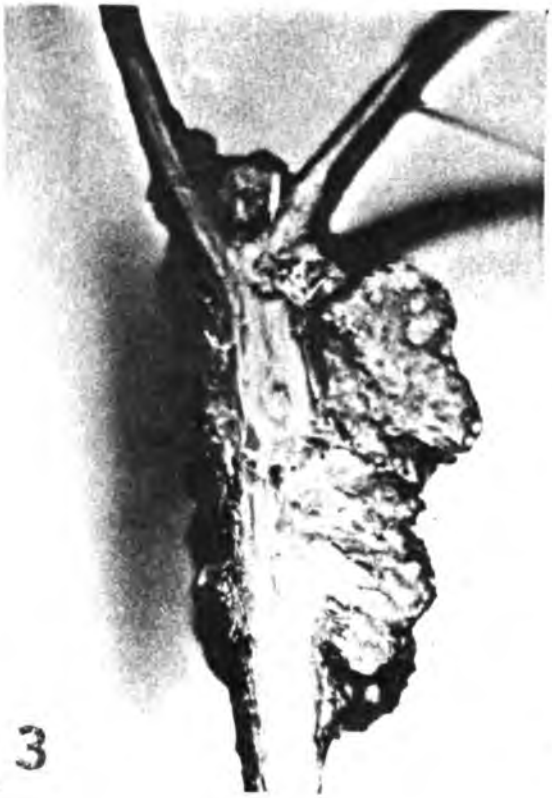
Figure 1. Microscopic section of a root sucker shoot tip prepared as an explant.

Figure 2. Root sphaeroblast with emerging shoot.

Figure 3. Longitudinal section of root sphaeroblast showing locations of origin within the root.

Figure 4. Acacia koa pollen grain.





20µ

4

flowers, and anthers with filaments. Pollen was also explanted. Koa pollen is a 16-celled compound grain typical of the Mimosoidae (Fig. 4) (Wodehouse 1935, Hyde and Adams 1958). All flower parts except peduncles were very difficult to surface sterilize.

Results

Effect of plant material source on decontamination

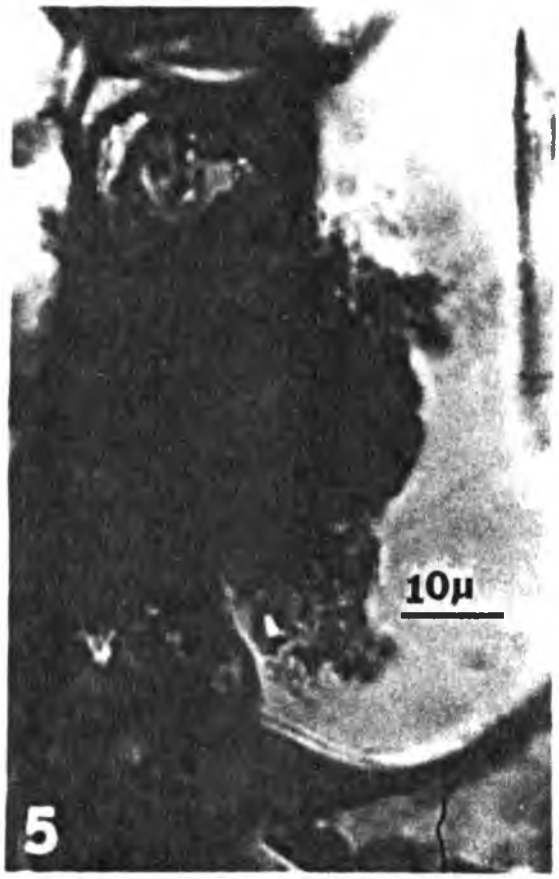
From a total of 851 explants only 228 or 27 percent were decontaminated and the high percentage of these successes occurred mostly with aseptically-grown seedlings, initially "clean" material such as cotyledons and embryos, or smooth material such as stems and petioles (Table 1). Most contamination that developed was bacterial and only appeared after a lengthy period. It appeared to arise internally in the tissue. Paraffin sections of several fresh root sprout shoot tips were made and stained for both gram positive and gram negative bacteria. Endogenous bacteria were gram negative and were found in several of these tips (Fig. 5). These bacteria were so situated (Fig. 6) as to be almost impossible to avoid either by excising or sterilization.

Success in surface sterilization was found to vary among different sources of explant material. Repeated attempts at surface sterilization of those explants containing endogenous bacteria reduced the potential for callus induction by damaging the surface tissues. This can be seen in the first three columns of Table 1.

Embryos and cotyledons did not require surface sterilization and were only rarely contaminated in culture. One-half of the embryos inoculated formed callus.

Figure 5. Bacteria erupting from an epidermal cell and infesting two adjacent cells in a root sucker shoot tip.

Figure 6. Bacteria infesting pro-vascular cells of a root sucker shoot tip only 20 cells distant from the apical meristem.


10μ
5


10μ
6

Most of the seedling material was taken from aseptically germinated seedlings, so was given only mild surface sterilization (3 to 5 minutes in 15 percent bleach). This probably accounted for the relatively high percentage of uncontaminated material which amounted to 35 percent of the inoculations made. Success in decontamination varied considerably with source of material, being 23 percent for tips, 16 percent for hypocotyls, and 50 percent for stems.

Probably because of the mild surface sterilization, 60 percent of the seedling explants that did not become contaminated in culture formed callus. Seedling stems which were most readily surface sterilized, formed callus very readily. A high 81 percent formed callus. Seedling tips were more difficult to surface sterilize successfully, but of those free of contamination, 69 percent formed callus.

Root sucker and adventitious shoot material were all field-collected and were much more likely to be contaminated. Most of the shoot tip explants from these sources required repeated treatments in bleach. This was reflected in the low success rate achieved in decontamination, 21 percent for the root sucker tips and 24 percent for the adventitious shoot tips. Excessive sterilization was reflected in the low successes achieved in callus induction from the successfully decontaminated explants.

Mature tissue was especially difficult to surface sterilize. Only 13 percent of it was freed of contamination. Flowers and flower parts were particularly difficult to sterilize, probably because of their complex structure. Phyllodinous tissue was generally readily

surface sterilized, but within a few days endogenous bacteria would erupt from within the explants. Mature tissue, except for inflorescence peduncles, did not form callus readily. Only one phyllode shoot tip formed callus. This may be because most of the phyllodinous material was explanted to media later found to be incapable of causing it to callus.

Effect of plant material source on callus regenerative capacity

The regenerative capacity of the callus induced from various explant sources, was strongly influenced by the source. The only calluses that formed adventitious shoots were those from ontogenetically juvenile material. Within this juvenile material, the only sources--except for a seedling hypocotyl--to retain regenerative capacity after callusing, were those containing primary, or meristematic tissues (Table 1).

Shoot tips, particularly those from aseptically grown, freshly germinated seedlings, were almost the only source of callus retaining totipotency. The only other calluses to regenerate shoots were one grown from a seedling hypocotyl and one from a meristematic portion of a root sphaeroblast.

Callus was more readily induced from juvenile material, than from mature tissue affording increased opportunities for regenerative capacity to be observed. However, the effect of explant source on callus induction was badly confounded by sterilization problems and by the effects of various media used in callus induction. The media tried for callus induction were not equal in function. Many were found to be incapable of callus induction.

Effect of media on callus induction

Although callus induction from seedling stems and hypocotyls occurred frequently with most common media, it was found to be very unusual for callus induction from seedling shoot tips to occur and extremely rare to occur from field-collected root sucker tips when these common media and supplements were used. Therefore, a large number of growth regulator modifications of several basal media were used in attempts to induce callus from the various explant sources. Callus induction was attempted on both solid, or agar media, and in liquid media. The basal media and growth regulator supplements used are listed in Table 2.

Initially, media 1 through 10 were tried as agar slants using aseptically grown seedling parts as explants. No seedling tips callused in these tests, only stems and hypocotyls did so. The media which induced callus most effectively were, in order of effectiveness, 4, 5, 8, 6, and 2. It was apparent that 2,4-D, together with coconut milk, readily induced callusing from seedling stems and hypocotyls.

While this first experiment was underway, several other media and explant sources were tested. The media included 47, 48, 49, and 50 in liquid form and additional agar slants of 4, 5, 6, and 8. The explants used included embryos, aseptically grown seedling parts, root sucker parts, and mature tree parts. One embryo callused in medium 49, Knudson's with coconut milk and adenine; no other callus induction occurred in this first group of liquid cultures. However, seedling shoot tip explants remained alive in both 47 (Vacin and Went) and in the Knudson's media (48 and 49) during a 2-month period. One of these

TABLE 2.--BASAL MEDIA AND GROWTH REGULATORS USED IN ATTEMPTS TO INDUCE CALLUS FORMATION FROM *ACACIA KOA* EXPLANTS

Reference numbers	Supplemented with[a]
	Murashige and Skoog (1962) Basal Medium
1	Not supplemented
2	10% CM
3	10% CM + 0.2 mg/ℓ 2,4-D
4	10% CM + 2.5 mg/ℓ 2,4-D
5	10% CM + 5 mg/ℓ 2,4-D
6	10% CM + 2.5 mg/ℓ NAA
7	3 (above) + 20 mg/ℓ adn
8	4 (above) + 20 mg/ℓ adn
9	5 (above) + 20 mg/ℓ adn
10	6 (above) + 20 mg/ℓ adn
11	1% CM
12	5% CM
13	15% CM
14	1% CM + 1.25 mg/ℓ 2,4-D
15	5% CM + 1.25 mg/ℓ 2,4-D
16	10% CM + 1.25 mg/ℓ 2,4-D
17	15% CM + 1.25 mg/ℓ 2,4-D
18	1% CM + 2.5 mg/ℓ 2,4-D
19	5% CM + 2.5 mg/ℓ 2,4-D
20	15% CM + 2.5 mg/ℓ 2,4-D
21	1% CM + 5 mg/ℓ 2,4-D
22	5% CM + 5 mg/ℓ 2,4-D
23	15% CM + 5 mg/ℓ 2,4-D
24	0.5% CM + 2.5 mg/ℓ 2,4-D
25	5 mg/ℓ C
26	5 mg/ℓ C + 2.5 mg/ℓ B
27	2.5 mg/ℓ C + 5 mg/ℓ B
28	5 mg/ℓ C + 5 mg/ℓ B
29	0.2 mg/ℓ 2,4-D, + 5 mg/ℓ C + 5 mg/ℓ K
30	1 mg/ℓ each IAA, NAA, 2,4-D, C, B, D, K, Ben, Z + 2 mg/ℓ GA
31	30 (above) without GA
32	30 (above) with BT instead of Ben
33	31 (above) with BT instead of Ben
34	1 mg/ℓ each IAA, NAA, 2,4-D, C
35	1 mg/ℓ each B, D, K, Ben, Z
36	1 mg/ℓ each IAA, NAA, 2,4-D, IBA, C, B, D, Ben, BT, K
37	0.2 mg/ℓ each IAA, NAA, 2,4-D, IBA, C, 1 mg/ℓ each B, D, Ben, BT, K
38	0.8 mg/ℓ each IAA, NAA, 2,4-D, IBA, C, 1 mg/ℓ B, D, Ben, BT, K
39	0.6 mg/ℓ each IAA, NAA, 2,4-D, IBA, C 1 mg/ℓ B, D, Ben, BT, K

TABLE 2. (Continued) BASAL MEDIA AND GROWTH REGULATORS USED IN ATTEMPTS TO INDUCE CALLUS FORMATION FROM *ACACIA KOA* EXPLANTS

Reference numbers	Supplemented with[a]
	Schenk and Hildebrandt (1972) Basal Medium
40	Not supplemented
41	0.2 mg/ℓ 2,4-D + 2 mg/ℓ C + 0.1 mg/ℓ K
42	41 (above) + 10% CM
43	0.2 mg/ℓ NAA
44	0.2 mg/ℓ K
45	1 mg/ℓ NAA, 0.5 mg/ℓ K
46	2.5 mg/ℓ NAA, 1.0 mg/ℓ K
	Vacin and Went (1949) Basal Medium
47	Not supplemented[b]
	Knudson (1946) Basal Medium
48	Not supplemented[b]
49	20% CM + 20 mg/ℓ adn[b]
	White (1963) Basal Medium
50	10% CM
51	10% CM + 2.5 mg/ℓ 2,4-D
	Nitch and Nitch (1969) Basal Medium
52	Not supplemented

[a] See page xiv for growth regulator abbreviations.

[b] Iron substituted at Murashige and Skoog (1962) level of Fe $SO_4 \cdot 7H_2O$ + Na_2EDTA

tips grown in medium 47 for 2 months was then placed in liquid medium 41 and 2 months later had formed callus as well as two sprouts from the original organ (Fig. 7).

One phyllodinous shoot tip formed callus on medium 6, which contained napthaleneacetic acid. This was the only callus to form from any such material. This shoot tip had been collected from a flushing, rapidly growing branch tip.

Flowers and flower parts were placed on agar slants of media 1, 2, 3, 50, 51, and 52, but none of this material was induced to callus.

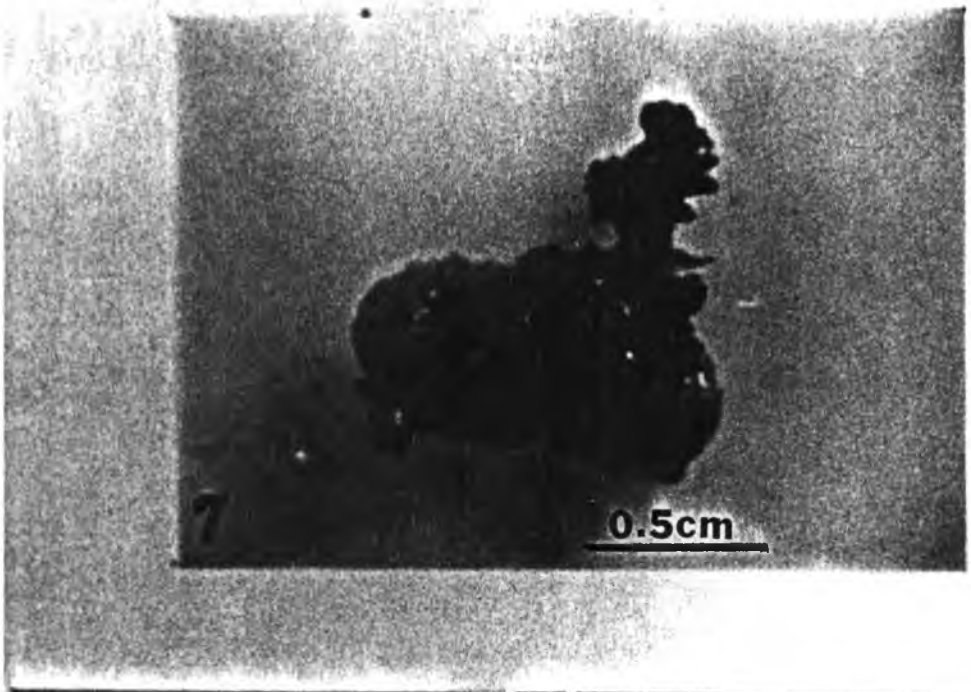
When it became apparent that callus induction was frequent with media containing 2,4-D and coconut milk, a series of factorial tests of various concentrations of 2,4-D and coconut milk were made. Media 11 to 23 and media 3, 4, and 5 were prepared in both liquid form and as agar slants. These media were inoculated with root sucker tips, cotyledons, seedling leaflets and petioles, seedling tips, and seedling stems.

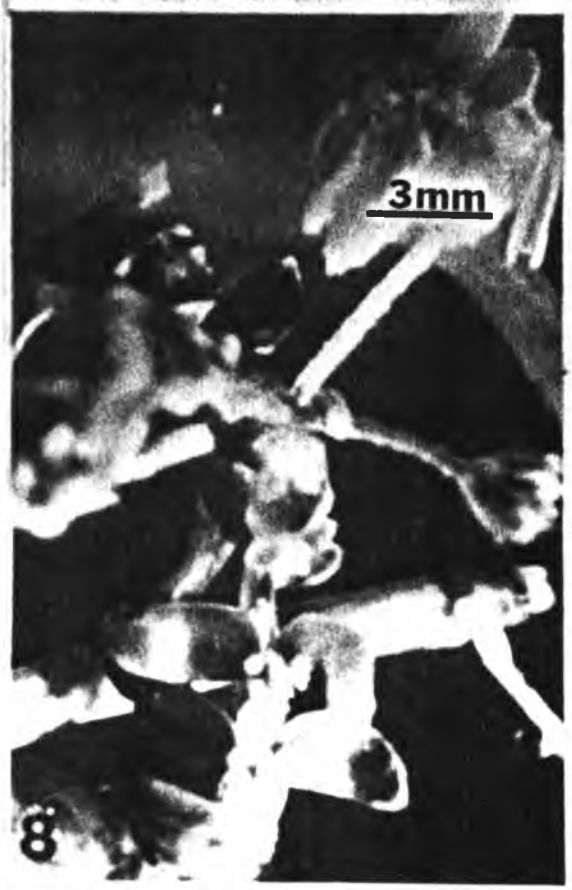
Root sucker tips in liquid media 22, 5, and 23, which had the highest levels of 2,4-D, became swollen, but did not form callus. Cotyledons did not react to any treatment. Two of five seedling tips formed callus on solid media 20 and 23, which had 2,4-D and a high level of coconut milk--15 percent. Seedling leaflets and petioles callused readily on media 11, 12, and 13 which contained only coconut milk (Fig. 8). They did not form callus on media containing 2,4-D. This indicated the variation in reaction that existed between sources of explants. The seedling tips required 2,4-D to form callus, but the leaflets did not callus with 2,4-D present.

Figure 7. Callus with two sprouts from the original seedling shoot tip. This formed and grew in liquid medium 41 (Table 2).

Figure 8. Callused seedling leaflets and rachises on medium 2 (Table 2).

Figure 9. Embryoids formed from seedling stem callus in medium 14 (Table 2).


7
0.5cm


3mm
8


0.5cm
9

Seedling stem callus formed readily in all the solid and in liquid media containing 2,4-D, but not in those containing only coconut milk. Stem tissue formed callus on media with 1 percent coconut milk (media 14, 18, and 21) and roots were formed after 2 to 3 months in the media. A stem callus in liquid medium 14 formed a cell suspension or mass of individual cells and cell clumps after 3 months in culture. Cell clusters in this suspension also formed roots and eventually rooted embryoids--structures with both a root and a shoot-like formation (Fig. 9).

Root sucker tips, stems, and leaflets were inoculated on agar slants and liquid culture of media 40, 43, 44, 45, and 46. These explants had been collected from adventitious shoots that had formed below a girdle on an air-layered root sucker. These shoots were relatively free of contamination because they had grown inside the air-layer wrapping. One of these root sucker tips callused on a slant of medium 45. No other tips formed callus, but two stems on agar slants of medium 43 and one in liquid 46 became swollen and eventually callused. This was the second instance in which field-collected shoot tip tissue reacted to napthaleneacetic acid rather than to 2,4-D as did laboratory-grown seedling material. It became apparent with this experiment that coconut milk was not essential for callus induction.

Other media which were found to induce callus quite well from seedling stem material were 25 and 28, which contained para-chlorophenoxyacetic acid and benzyladenine. These did not induce callus from other seedling parts, or root sucker parts, except for two root sucker stems. They contained no coconut milk, indicating that it

was not essential for callus induction.

Media 25 to 29 had been prepared to evaluate the use of high levels of auxin and a single cytokinin instead of coconut milk for callus induction. The p-chlorophenoxyacetic acid was substituted for 2,4-D because 2,4-D at high levels is known to cause genetic aberrations in cultures (M. Mapes, personal communication), inhibits shoot formation in some callus cultures (Murashige 1974b), and had been used in this manner by Schenk and Hildebrandt (1972) in their medium.

While experiments of callus induction were continuing, other experiments using media intended to cause differentiation of callus into organized structures were in progress. The details of these experiments are given in Chapter 3. Early results from these tests demonstrated that calluses derived from seedling shoot tips and hypocotyls could organize into shoots. But no organization of callus from stem tissue or leaves occurred. Juvenility of the explant sources and the presence of primary tissue in the explants seemed to be important to future differentiation of calluses formed from them. The only sources of juvenile explants in mature koa trees were root sucker and adventitious shoot tips. Since the objective was to clone these trees, this material was given increased attention.

From among 17 field-collected shoot tips that had not been discarded because of contamination, only two had been induced to callus. These had callused in media containing napthaleneacetic acid at levels of 1 and 2.5 mg/ℓ. Other tips had become swollen at high levels of 2,4-D although they had not callused.

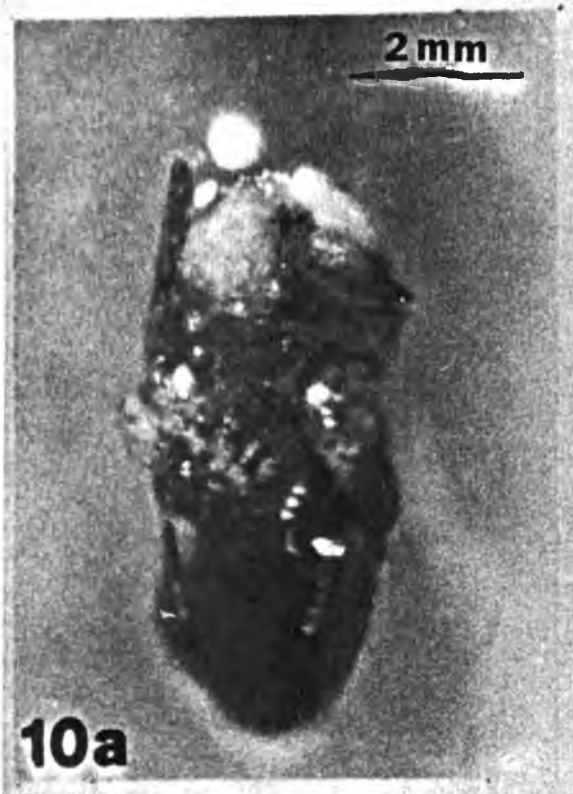
Results cited in the literature review indicated that combining growth regulators might enhance their functions. Improved growth and differentiation had been obtained by using two auxins and/or two cytokinins, so possibly by using three or four even more improvement would occur. As a test of this possibility, a medium containing four auxins, five cytokinins, and gibberellic acid (medium 30) was used for both callus induction and callus growth. The makeup of this medium was happenstance. The particular growth regulators used happened to be available at the time. This medium both greatly improved callus induction and greatly increased growth of existing calluses.

Almost any explant material tried, would form callus on medium 30. Root sucker tips and inflorescence peduncles that had not previously callused, did so on medium 30 (Fig. 10). Moreover, it was found later that shoot regenerative capacity was retained in calluses grown on the medium. Explants inoculated to the medium included all seedling parts except petioles, all root sucker parts including root sphaeroblasts, adventitious shoots, and inflorescence peduncles.

Attempts were made to improve upon the formulation of medium 30. These supplements are indicated by numbers 31 to 39 in Table 2. One of these, medium 32, induced callus well. It was used repeatedly in liquid and agar form with approximately 30 percent success in callus induction of uncontaminated root sucker tips and 10 percent success with root sphaeroblasts. Unfortunately, calluses induced on medium 32 showed little regenerative capacity. Only one root sphaeroblast callus and one root sucker tip callus initially formed on medium 32 formed shoots. The simple substitution of benzythiazolyloxyacetic

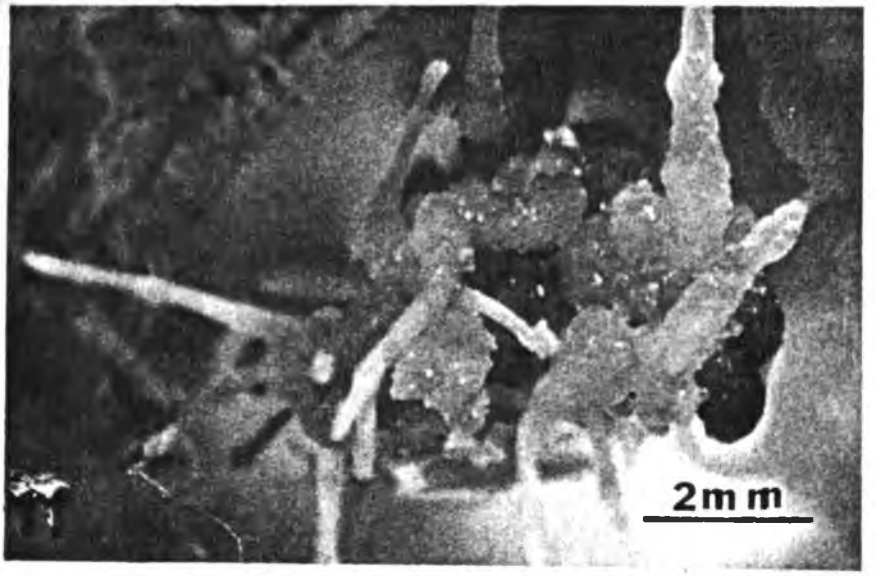
Figure 10. Root sucker shoot tip (a) and inflorescence peduncle (b) callusing on medium 30 (Table 2).

Figure 11. Roots formed from seedling stem callus on medium 14 (Table 2).



10a

10b



acid for benzylaminobenzimidazole seemed to reduce shoot regenerative capacity. In another instance, callus placed on the similarly supplemented medium 31 was so altered from callus kept on medium 30 that it died after placement on a medium intended to cause shoots to form, while the other callus that had been on medium 30 produced multiple shoots.

Two other modifications, numbers 38 and 39, were almost as effective in callus induction as medium 32, but there was no subsequent evidence of shoot regenerative capacity in calluses formed on them. Media with only auxins, or only cytokinins (34 and 35) did not induce callus. A medium with 5 mg/ℓ each of auxins and cytokinins (36) did not induce callus either, although those with the same auxins at slightly reduced levels (38 and 39) were very effective. Medium 37 with a very low amount of mixed auxins, did not induce callus, but induced shoot formation from callus. None of the modifications of medium 30 was as successful as medium 30 in inducing totipotent callus.

The media with which success was achieved in callus induction from various explants are listed in Table 3. It will be noted that medium 30 appears very frequently as the most suitable medium for a particular explant source.

Effect of media on callus shoot regenerative capacity

Only 12 of the 72 calluses grown were later caused to differentiate adventitious shoots (Table 1). As has been indicated, this was probably largely the result of the explant source rather than the medium used to induce callus formation.

TABLE 3.--MEDIA FOUND SUITABLE FOR CALLUS INDUCTION OF VARIOUS *ACACIA KOA* EXPLANT SOURCES

Explant source	Media[a]
Embryos	2s, 49ℓ
Seedling	
tip	30sℓ, 32sℓ, 38sℓ, 41ℓ, 20s, 23s
hypocotyl	4s, 3s
stem	30s, 4s, 5s, 3s, 8s, 14-20s, 25s, 28s
leaf	12s
petiole	30s, 11s, 12s
Root sucker	
tip	30sℓ, 32sℓ, 38sℓ, 45s
stem	30sℓ, 32sℓ, 38sℓ, 43s, 46ℓ, 25s, 28s
leaf	30sℓ
petiole	30sℓ, 32sℓ
root sphaeroblast	32s
Adventitious shoot	
tip	30ℓ, 38s
Mature tree	
phyllode tip	6s
peduncle	30s, 32s

[a] Media numbers refer to Table 2. Media are given in order of success, the first listed being the most successful in callus induction. s = agar (solid), ℓ = liquid, sℓ = both agar and liquid media were successful.

The callusing medium may also have had an effect on future differentiation of the callus. As was noted in the last section, one obvious effect was that stem calluses held for more than 3 months on 2,4-D media such as 4, 5, 8, and 21 of Table 2 would form roots (Fig. 11). Both White (1963) and Murashige (1974b) have noted that calluses that have formed roots generally do not form adventitious shoots. Therefore, the formation of roots that was caused by the medium was an effect of the medium on totipotency because entire plants could not be formed once roots had formed.

However, all calluses formed on 2,4-D containing media were not necessarily incapable of regenerating organized shoots. Among the six totipotent seedling tip calluses, four were initiated in medium 41, one in 30, and one in 7. All these contained 2,4-D, in low amounts. A hypocotyl callus also produced a shoot. This callus formed on medium 4 and was on this relatively high 2,4-D medium for 4 months.

Two of the root sucker tip calluses that formed shoots were originally induced in medium 30, the other in medium 32. The root sphaeroblast callus that formed shoots was induced to callus in medium 32. And the adventitious shoot tip that differentiated was induced initially in medium 30.

Evidence of shoot regeneration capacity among the calluses was so rare that the effect of the callus inducing media, if any, was not determined. The results suggested, however, that media 30 and 41 were the most likely media to induce callus capable of shoot regeneration.

Discussion

The difficulty of removing endogenous bacteria by surface sterilization proved to be a major obstacle in tissue culture propagation of the species. Only 27 percent of the explants inoculated were successfully freed of contaminants (Table 1). Among those that were uncontaminated, only 32 percent were induced to form callus. The explants were killed in repeated attempts to kill the bacterial contaminants.

The endogenous bacteria in field-collected material may have prevented callus induction in many instances by using up nutrients and growth regulators. Even when callus was induced, the bacteria in the callus may have reduced the possibility of shoot regeneration. This is suggested by the relatively high levels of successful callus induction and shoot regeneration achieved with laboratory-grown, essentially uncontaminated material, as compared with ontogenetically similar material collected in the field.

Continued work on this problem should be aimed at collecting only flushing, rapidly growing material in the field and excising from its tips only a few cells of the apical meristem. Such material has frequently outgrown the bacteria and viruses (Murashige 1974b). Unfortunately, such procedures create an additional problem, because it has been found that very small explants with few cells are much less likely to survive in culture than larger sections (Murashige 1974b). However, these very small sections of rapidly dividing cells would be most likely to be free of the endogenous bacteria prevalent in koa.

The findings of most other workers with trees (Ball 1950, Winton and Huhtinen 1976, Pierik 1975) that juvenility is a key factor in regeneration from callus just as it is in whole cuttings (Hartmann and Kester 1975) are further borne out with koa. The regenerative capacity of callus and callus induction itself appeared to be strongly influenced by juvenility, or lack of it.

The root suckering characteristic of koa provides a ready supply of juvenile material from the roots of mature trees which can be induced to form callus with the ability to regenerate plants. If this were not the case, tissue culture propagation of koa would be limited to seedling material just as it presently is with most other trees (Pierik 1975).

The development of the callus inducing medium 30 (Table 2) was an important breakthrough towards the cloning of mature koa trees. It provided a means of inducing callus from field-collected material that had not callused on any more conventional medium. The reasons why a mixture of growth regulators should induce callus when used alone and they would not, was not investigated intensively.

But the comparisons that were made indicated that callus induction definitely occurred much more readily in the presence of 1 mg/ℓ each of four or five auxins and five cytokinins than in the presence of 4 to 5 mg/ℓ of a single auxin together with 5 mg/ℓ of a single cytokinin.

Summary

Callus with the capacity to regenerate new shoots was induced most readily from seedling and root sucker shoot tips on agar or in

liquid media containing Murashige and Skoog's (1962) salts and vitamins and a mixture of 1 mg/ℓ each of indoleacetic acid, naphthaleneacetic acid, para chlorophenoxyacetic acid (C), 2,4 dichlorophenoxyacetic acid (2,4-D), benzyladenine, dimethylallyladenine, benzylaminobenzimidazole, kinetin (K), and zeatin and 2 mg/ℓ of gibberellic acid.

Such callus was also induced from seedling shoot tips in Schenk and Hildebrandt (1972) basal medium with 0.2 mg/ℓ 2,4-D, 2 mg/ℓ C, and 0.1 mg/ℓ K.

Many other media were used successfully to induce callus from other sources of explants, but, with the exception of one hypocotyl culture, only explants of juvenile parts containing primary tissue produced callus capable of regenerating shoots, and the above media were the most common ones that induced callus in such explants.

Stem tissue of both seedlings and root suckers callused readily on media containing several different growth regulators. Stem calluses, however, were found to only regenerate roots, or embryo-like structures called here embryoids. The embryoids and the rooted calluses did not form adventitious shoots capable of regenerating whole plants, so did not demonstrate totipotency.

Koa plant tissue, particularly that collected in the field, contained endogenous bacteria and was very difficult to surface sterilize for aseptic culturing. Problems with contamination and the effects of over sterilization influenced all experiments and results.

CHAPTER 2

GROWTH OF CALLUS AND CELL SUSPENSION CULTURES OF *ACACIA KOA* TISSUE

Introduction

If mass propagation of plants by the callus or cell suspension techniques of tissue culture is to be successful, then large amounts of callus have to be grown quickly. This is necessary not only for the obvious reason that with more available callus, there should be more potentially totipotent cells, but also because most plant tissue loses its regenerative capacity rapidly during culture (Murashige 1974b, Yeoman 1973). Ball (E. A. Ball, personal communication), for example, uses only "primary" callus, callus transferred directly from the induction medium, to propagate *Sequoia sempervirens* because he finds it quickly loses the ability to regenerate. Callus must be increased and maintained in conditions that will reduce this tendency.

Experiments intended to increase the growth of koa tissue in culture were carried out concurrently with experiments on callus induction and on the regeneration of shoots from callus. In fact, in many instances all three objectives were combined in one factorial experiment of various levels of auxins and cytokinins, because it is the levels of these ingredients, more than any other factor, that control callus induction, callus growth, and organogenesis of callus (Gamborg and Wetter 1975, Murashige 1974a and 1974b, Yeoman 1973).

The structure of the callus that is grown is also very important to its future regenerative capacity. Callus may be friable, or firm; smooth and rounded, or lumpy. This can be greatly influenced by both

basal media and growth regulators, as well as by laboratory conditions (Murashige 1974b). With many plants, the growth of cells containing many chloroplasts giving the callus a rich green color is aimed for, because this type of tissue is usually most capable of shoot regeneration (M. Mapes, personal communication). In cell suspension cultures, globular rapidly dividing cells with little vacuolation are sought (Street 1973b). These are the type most likely to show embryogenesis.

Callus growth experiments with koa covered a broad range of basal media, growth regulators, and environmental conditions. Some experiments on agar media were more amenable to growth measurements than others. The callus was grown with the ultimate objective of regenerating shoots, so was not submitted to destructive testing or measurement.

Literature Review

There have been several attempts at measurement of callus and cell suspension growth. Caplin (1947) did an extensive study of tobacco callus growth on agar. He found that the growth consisted of the extension of "knobs" that formed near the explant surface, so that a flattened, lumpy structure rather like a cauliflower head formed. The original callus explant became a discolored piece at the center of the the new callus. Others (Henderson, Durrell, and Bonner 1952, Straus and La Rue 1954) found a similar growth pattern in callus grown from other plant tissues.

Each "knob" of callus may have a different growth rate (Caplin 1947). The callus becomes heterogeneous during growth so that

different parts of it, when they are subcultured, react in a different manner to the same medium (Caplin 1947). The great variability among subcultures of a single callus has resulted in a scarcity of publications on growth analyses of callus cultures (Yeoman 1973).

Cell suspension cultures have also shown variability (Liau and Boll 1971, Nash and Davies 1972, Durzan and Lopushanski 1975). Pigmented and unpigmented cells occur in the same culture (Blakely and Steward 1961, Steward, Israel, and Mapes 1968). The degree of cytochrome oxidase varies between cell clumps (deJong, Jansen, and Olson 1967), and rates of protein synthesis as well as the locations of amino acids vary among cells varying in amounts of aggregation (Verma and Van Huystee 1970).

Methods of measuring growth of callus have usually been by weight. Fresh weight and dry weight were used by Caplin (1947), Henderson, Durrel, and Bonner (1952), and Straus and La Rue (1954). In cell suspension cultures, most measurements have been made by haemostat counts of cell number per unit volume, although some studies (Filner 1965) have included dry weight, or even change in DNA content over time (Nash and Davies 1972). All methods used to evaluate growth were destructive.

Cell aggregation or separation in culture is closely related to media constituents. Torrey and Reinert (1961) for example, controlled cell separation by reducing the concentration of coconut milk in the medium. Torrey, Reinert, and Merkel (1962) controlled cell separation by changing auxin levels in the medium, and Simpkins, Collin, and Street (1970) markedly influenced cell separation by varying auxin and

cytokinin levels.

In agar culture, the condition of the callus depends a great deal on the media constituents and the environment in which it is grown. Firm callus can be changed to friable callus by changing the levels of auxin and coconut milk (Blakely and Steward 1964). White callus can be made green by the addition of cytokinin (Stetler and Laetsch 1965). Winton (1970) produced firm, white callus by keeping it in total darkness. Steward (1970) caused callus to form cell suspensions by placement in liquid medium with reduced calcium to reduce the formation of calcium pectate in the middle lamella.

Callus growth may be increased by the addition of auxin, cytokinin, both auxin and cytokinin, or other growth regulators to the basal medium, depending on the plant tissue being cultured and the source of the callus in the plant (Yeoman 1973). Generally, a high auxin level promotes cell division and enlargement, and the presence of cytokinin at moderate levels promotes cell division (Yeoman 1973). With many tissues other additives such as myo-inositol, glycine, or casein hydrolysate have a measurable effect on growth (Yeoman 1973). Coconut milk has a cytokinin-like effect (Van Overbeek et al. 1941).

The levels and combinations of growth regulators that best promote callus growth, as well as differentiation, have been examined using factorial experiments (Steward 1970, Yeoman 1973). Generally, such experiments have been designed to compare the effects of four to five levels of auxin concentration against four to five levels of coconut milk (Steward, Caplin and Millar 1952) or of a cytokinin (Murashige 1974b, Gamborg et al. 1976). Generally, when working with

a new species, a first approximation is made by repetitive empirical tests to discover what basal media and growth regulators cause rapid growth (Gamborg et al. 1976). Later, refinements are made to the initial findings by factorial experiments (Gamborg et al. 1976).

The possibility, or even desirability of attempting to measure callus growth is debatable. Gamborg et al. (1976) stated: "Frequently much time has been spent on developing procedures for growing callus of a given plant, but with limited success. It should be clearly understood that getting fast-growing cultures with a cell generation (doubling) time of, for example, 24 to 48 hours, involves not only suitable media and laboratory environment, but also careful timing of subculture. In addition, a process of cell selection is taking place. A failure to recognize these points can easily lead to a lengthy search for a 'super' medium. The results have been an array of 'new' media, which is an unfortunate development.----the continued appearance of new media,---has introduced a new variable in comparative plant cell research."

Materials and Methods

Acacia koa callus and cell suspension growth experiments were carried out in two different laboratories, both kept at a constant 25° C. Most of the experiments were done in one of the two laboratories and consisted of plating out callus on agar in petri dishes, growing callus on agar slants, and growing either pieces of firm callus or cell suspensions in liquid media in erlenmeyer flasks, t-tubes, or nipple flasks. In the other laboratory, the only growth experiments were done on agar slants, or in erlenmeyer flasks.

The techniques used for studies of growth of callus and cell suspensions in culture are very well stated in Yeoman (1973) and Street (1973b). These techniques consist of the placement of equal sized callus explants on slants of solidified (agar) media, or totally submerged in liquid media kept constantly in motion, or periodically immersed in liquid media in t-tubes or nipple flasks following the methods of Steward, Caplin, and Millar (1952). At some point, a cell suspension culture begins as a callus culture and eventually separates naturally, or because of agitation, into cell aggregates and individual cells (Street 1973b).

The experiments were conducted in continuous light. Photon flux densities in the photosynthetically active range which were used were: for agar slants, from 30 to 66 $\mu E\ m^{-2}sec^{-1}$; for agitated erlenmeyer flasks, from 16 to 24 $\mu E\ m^{-2}sec^{-1}$; for t-tubes, from 30 to 47 $\mu E\ m^{-2}sec^{-1}$; and for nipple flasks, 42 $\mu E\ m^{-2}sec^{-1}$. These levels of light were in the range of those used by Kakade and O'Connor (1977) for experiments on growth of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] callus. They were obtained using "Cool-white" (Westinghouse) and "Gro-lux" (Westinghouse) fluorescent lamps. Agar slants were enclosed in a light-tight box for experiments of callus growth in darkness.

Cultures in erlenmeyer flasks of liquid medium were agitated at either 60 or 100 rpm on two separate gyro-rotary shakers. Cultures in t-tubes were rotated at 1 rpm and nipple flasks were rotated at 2 rpm. No experiments were undertaken to compare growth of cell suspensions at different rates of agitation, but more rapid growth appeared to

occur in those cultures in erlenmeyer flasks that were agitated at 100 rpm rather than at 60 rpm.

All of the callus growth experiments on agar media were made by plating two or three more or less spherical pieces of callus onto agar media in slants or petri dishes. In most experiments calluses from two or three different sources were inoculated on each of two or more slants or plates in order to replicate experimental material. In a few experiments the individual slants or plates were the replications and all material on any one slant or plate was from the same source.

The initial size and growth of the callus explants was determined by comparing their diameters as seen from above in the plane of the agar surface with circles of measured diameter drawn on a piece of clear acetate film. Koa callus growth in this plane was judged to be most rapid, as was also that of tobacco callus as determined by Caplin (1947). Growth between periods of measurement was treated as a straight line relationship of diameter increase per day in order to estimate the number of days to doubling in diameter which was sometimes used as the comparison measurement.

Growth of callus in liquid media, or of cell suspensions, was simply visually estimated. No measurements were made either of the material originally explanted, or of material later removed from the media. However, differences among treatments were usually so large that simple categorization of growth as excellent, good, fair, or poor provided useful information.

Destructive measurements of callus and cell suspension growth were avoided because the material grown was all used for other

experiments of differentiation of callus.

Most of the callus growth experiments were designed as factorial experiments of the randomized block design. In these experiments growth was most commonly compared at different levels of auxins and coconut milk or auxins and cytokinins. Other factorial experiments were comparisons of different auxins and different cytokinins, different levels of basal media additives such as myo-inositol and sucrose, or media pH.

Basal media used in the callus growth experiments were those of Murashige and Skoog (1962) and Schenk and Hildebrandt (1972). The formulations of these media are given in Appendix A. All experiments of growth on media gelled with agar were made on Murashige and Skoog's basal medium or modifications of it. Agar was added at 8 g/ℓ. Experiments made with liquid media utilized either Murashige and Skoog's or Schenk and Hildebrandt's media.

Variations of Murashige and Skoog's basal medium included elimination of ammonium nitrate, variation in level of sucrose and pH, and the level of myo-inositol was sometimes increased from 100 to 1000 mg/ℓ. Schenk and Hildebrandt's basal medium was used as it appears in Appendix A, except that myo-inositol was reduced from 1000 mg/ℓ to 100 mg/ℓ in some experiments.

The growth regulators and other growth influencing substances added to the basal media appear in the list of abbreviations (page xiv). These were added in various amounts and combinations as will be brought out in the section on results.

A complete list of all media given trial for increasing growth of

callus and cell suspensions is given in Appendix B. The media are rated according to their effectiveness.

Results

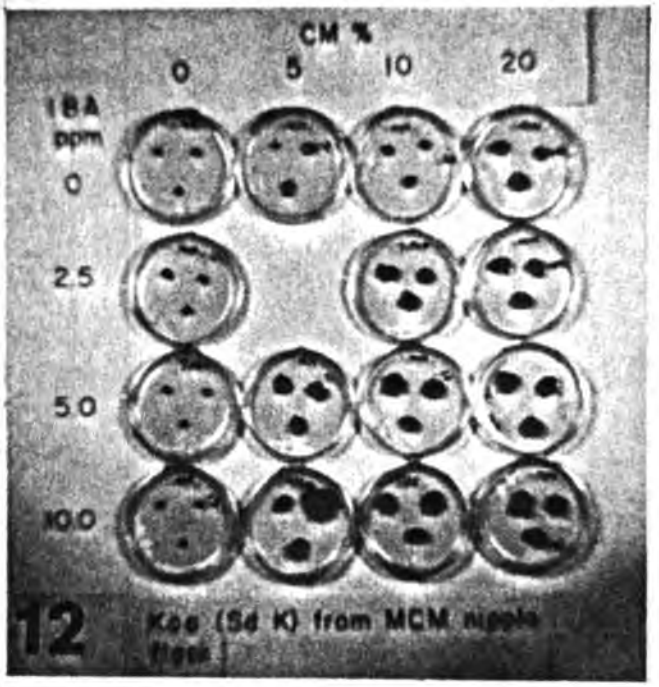
Factorial experiments of callus growth on agar media

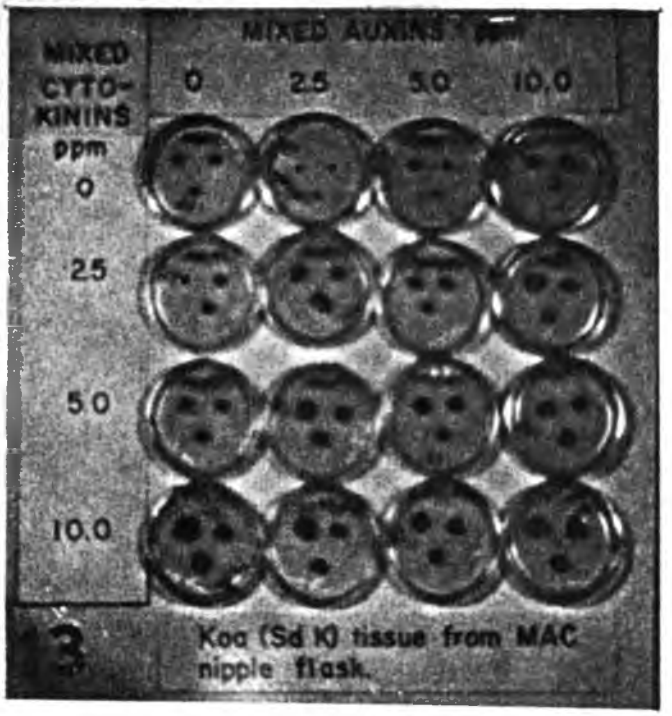
Initial experiments of callus induction from koa seedling parts were made using Murashige and Skoog's (1962) medium modified with 2,4-dichlorophenoxyacetic acid (2,4-D), napthaleneacetic acid (NAA) and coconut milk. Calluses were induced from seedling stems on several growth regulator combinations, but were observed to grow most rapidly on media containing 2,4-D and coconut milk. Because of this, numerous experiments were subsequently conducted to determine which levels of these two substances caused the most rapid growth of callus from different sources, and later what other auxins and cytokinins could be substituted for the 2,4-D and coconut milk.

Although it was not one of the original experiments, a comparison of levels of indolebutyric acid (IBA) and coconut milk (CM) on the growth of seedling tip callus (Fig. 12) clearly shows a typical experimental layout and results. In this experiment, three pieces of callus obtained from a liquid culture of a seedling tip identified as SdK were plated out on each of 16 petri dishes containing Murashige and Skoog's basal medium to which had been added the indicated amounts of indolebutyric acid in ppm (or mg/ℓ) and coconut milk in percent. It is clear that the largest average diameter occurred in the treatment with 10 ppm indolebutyric acid and 5 percent coconut milk. It can also be seen that no growth occurred in the absence of coconut milk and almost none, except in the 20 percent coconut milk treatment,

Figure 12. Factorial test of indolebutyric acid and coconut milk levels on callus growth.

Figure 13. Factorial test of four levels each of mixed auxins and mixed cytokinins on callus growth.


CM %
0
5
10
20
IBA ppm
0
2.5
5.0
10.0
12
Koa (Sd K) from MCM nipple flask


MIXED AUXINS ppm
0
2.5
5.0
10.0
MIXED CYTO-KININS ppm
0
2.5
5.0
10.0
13
Koa (Sd K) tissue from MAC nipple flask.

in the absence of indolebutyric acid. The photograph was taken 10 weeks after inoculation of equal sized explants. One treatment became contaminated and was discarded.

The first experiments undertaken were comparisons of callus growth in diameter at 2,4-D levels of 0, 1.25, 2.5, and 5.0 mg/ℓ and coconut milk levels of 1, 5, 10, and 15 percent. These experiments, as with all others of callus growth on agar, were made using Murashige and Skoog's basal medium. Six replications of seedling stem callus were used in the first two of these experiments. There were highly significant differences in growth caused by the two growth regulators, but no significant interaction between them. The best average growth occurred on the medium with 2.5 mg/ℓ 2,4-D and 5 percent coconut milk (No. 1, Table 4).

The results of a third experiment made with seedling tip callus using three replications indicated the best growth at 2.5 mg/ℓ 2,4-D and 15 percent coconut milk. Callus from seedling leaflets and rachises in the fourth experiment with these same comparisons only grew on the media lacking 2,4-D. The best average growth was on the 0 mg/ℓ 2,4-D, 15 percent coconut milk treatment. The four experiments together indicated that callus growth was affected by its original source as well as by the growth regulators supplied.

Lower levels of 2,4-D were used in a 5 x 5 factorial in which three replications each of white and of green seedling stem callus were grown on basal medium containing 0, 0.2, 0.4, 0.8, or 1.6 mg/ℓ of 2,4-D and 0, 1.25, 2.5, 5, or 10 percent coconut milk. In this experiment the most rapid growth of white callus was on the 0.8 mg/ℓ

TABLE 4.--GROWTH RATES OF THE MOST RAPIDLY GROWING CALLUSES ON THE MOST SUCCESSFUL GROWTH PROMOTING GROWTH REGULATOR COMBINATIONS FROM FACTORIAL EXPERIMENTS USING MURASHIGE AND SKOOG AGAR BASAL MEDIUM

Number	Factorial test[a]	Callus source	Fastest growth on	Estimated days to double diameter	Fast-grown callus condition
1	2,4-D @ 0, 1.25, 2.15, 5.0 mg/ℓ	Seedling stem	2,4-D 2.5, CM 5%	96	Firm, green
	CM @ 1, 5, 10, 15%	Seedling tip	2,4-D 2.5, CM 15%	60	Soft, white
		Seedling leaves	2,4-D 0, CM 15%	-	Soft, white
2	2,4-D @ 0, 0.2, 0.4, 0.8, 1.6 mg/ℓ	White seedling stem	2,4-D 0.8, CM 2.5%	17	Firm, white
	CM @ 0, 1.25, 2.5, 5, 10%	Green seedling stem	2,4-D 0.8, CM 10%	24	Firm, green
3	CM @ 0, 1.25, 2.5, 5%	Seedling tip	B 5.0, CM 2.5%	13	Soft, green
	B @ 0, 2.5, 5.0, 10 mg/ℓ	Seedling stem	B 5.0, CM 2.5%	12	Soft, green
4	NAA @ 0, 1.25, 2.5, 5.0 mg/ℓ	Stem callus	N 2.5, B 5.0	74	Firm, green
	B @ 0, 1.0, 5.0, 10.0 mg/ℓ				
5	2,4-D @ 0, .25, 0.5, 1.0 mg/ℓ	Seedling tip	2,4-D 0.25, B 10.0	30	Firm, green
	B @ 0, 1.0, 5.0, 10.0 mg/ℓ				
6	C @ 0, 2.5, 5 mg/ℓ	White seedling stem	C 2.5, B 5.0	20	Soft, green
	B @ 0, 2.5, 5 mg/ℓ	Green seedling stem	C 2.5, B 5.0	27	Soft, green
7	SU[b] @ 0, 10, 20, 30, 40 g/ℓ	Seedling tip	SU 20, CM 10%	100	Soft, green
	CM @ 0, 10, 20, 30, 40%	Root sucker tip	SU 30, CM 30%	29	Soft, white
8	IBA @ 0, 2.5, 5.0, 10.0 mg/ℓ	Seedling tip	IBA 10, CM 5%	19	Firm, green
	CM @ 0, 5, 10, 20%				
9	Aux.[c] @ 0, 2.5, 5.0, 10.0 mg/ℓ	Seedling tip (liquid)	Aux. 2.5 Cyt. 5.0	31	Soft, green
	Cyt.[d] @ 0, 2.5, 5.0 10.0 mg/ℓ	Seedling tip (agar)	Aux. 5.0 Cyt. 5.0	14	Soft, green

[a] Abbreviations for growth regulators are on page xiv.

[b] SU means sucrose.

[c] Aux. means mixed auxins, equal amounts of IAA, IBA, C, NAA, 2,4-D.

[d] Cyt. means mixed cytokinins, equal amounts of B, D, Ben, BT, K.

2,4-D and 2.5 percent coconut milk medium. The best growth of the green callus was also at 0.8 mg/ℓ 2,4-D, but was at 10 percent coconut milk. The white callus grew more rapidly (No. 2, Table 4). The presence of chlorophyll seemed to influence growth because in this test both the white and the green calluses were from the same source.

Next, an experiment was made to determine if koa callus could be grown with coconut milk substituted for the auxin normally used and benzyladenine, a cytokinin, substituted for the coconut milk. This was laid out as a factorial with two replications each of seedling tip callus and two of seedling stem callus. The levels of the growth regulators used were coconut milk: 0, 1.25, 2.5, and 5 percent and benzyladenine 0, 2.5, 5.0, and 10.0 mg/ℓ. Only one treatment produced growth, the treatment with 2.5 percent coconut milk and 5 mg/ℓ benzyladenine. All replications of both sources grew well on this one combination, forming dark green callus. But when the medium was made again, the results could not be duplicated. Coconut milk was apparently unable to supply the auxin requirement.

A factorial experiment of napthaleneacetic acid at 0, 1.25, 2.5, and 5.0 mg/ℓ and benzyladenine at 0, 1.0, 5.0, and 10.0 mg/ℓ using three replications of white stem callus caused necrosis of all explants except one with 2.5 mg/ℓ of the auxin and 5.0 mg/ℓ of the cytokinin. This became pale green. Growth was poor in all treatments.

Another factorial experiment was made to measure growth on media modified with 2,4-D at 0.25, 0.5, and 1.0 mg/ℓ and benzyladenine at 0, 1.0, 5.0, and 10.0 mg/ℓ. This experiment was intended to test the possibility of increasing growth on media containing benzyladenine at

levels that had been found to promote differentiation by supplying a little auxin along with the cytokinin. Callus of four different seedling tip sources was used as explants of three replications each. Growth occurred only in the 5 and 10 mg/ℓ benzyladenine treatments that contained 2,4-D. The treatment with 0.25 mg/ℓ 2,4-D and 10.0 mg/ℓ benzyladenine produced the best growth from three of the four callus sources. This experiment indicated that callus growth could be increased by adding 2,4-D to benzyladenine media, but no differentiation occurred from any of these cultures after growth on these media, so the 2,4-D may have inhibited it.

These and other tests that were run are summarized in Table 4. The tests numbered 1 to 5 have been described. In these tests, growth of seedling stem callus was usually found to be much more rapid than growth of callus derived from seedling tips. There was a large variation between and within the experiments in the time required for callus diameter to double.

In experiment 6 (Table 4), para-chlorophenoxyacetic acid (C) was substituted for 2,4-D, and benzyladenine for coconut milk. Rapid growth occurred in the media containing benzyladenine at 5.0 mg/ℓ and C at either 2.5 or 5.0 mg/ℓ, but seedling stem callus growth was not as rapid as on 2,4-D coconut milk media.

Another test (No. 7, Table 4), was made to examine the combined effect of sucrose levels and coconut milk levels on callus growth. For this test calluses grown from a seedling shoot tip and a root sucker shoot tip were used. The seedling tip callus was replicated four times and the root sucker tip three times at each level. Three

of the four seedling tip replications, and one of the three root sucker tip replications were obtained from liquid cultures and grew very poorly when placed on agar. The 100 days required to double diameter reflects the poor growth. The faster growth of the root sucker tip material may be because most of it was transferred from agar to agar. Liquid to agar transfers often resulted in reduced growth. Other results of this experiment, which are not shown, indicated that growth of the calluses was generally similar throughout a wide range of sucrose and coconut milk levels, but was completely stopped at levels of 40 g/ℓ of sucrose, or at 0 levels of either sucrose or coconut milk.

Experiment number 8 (Fig. 12) consisted of two sets of petri dishes, each with three replications of seedling tip callus. Growth at 5.0 mg/ℓ indolebutyric acid and 10 percent coconut milk nearly equalled that shown in Table 4 for 10.0 mg/ℓ IBA and 5 percent coconut milk.

Figure 13 depicts one block of factorial test 9. It had been determined prior to the establishment of this experiment that a mixture of 1 mg/ℓ each of indoleacetic acid, napthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, p-chlorophenoxyacetic acid, benzyladenine, dimethylallyladenine, kinetin, benzylaminobenzimidazole, and zeatin, with 2 mg/ℓ gibberellic acid induced very rapid growth of callus tissue of every explant source tried. The experiment results shown in Figure 13 (No. 9, Table 4) was an attempt to determine callus growth differences at various levels of a mixture of five auxins and a mixture of five cytokinins. The treatments were made by proportioning

stock solutions containing the auxins or cytokinins (Table 4) used in these mixtures into the basal media to which 2 mg/ℓ of gibberellic acid had been added.

There were replications of this experiment, two with seedling tip callus from liquid cultures and two with similar callus from agar cultures. The calluses from liquid grew erratically, but those from agar grew in relation to auxin-cytokinin levels. These grew well at a level of 2.5 mg/ℓ of auxins and 5.0 mg/ℓ of cytokinins. They grew best at 5.0 mg/ℓ of each. It was subsequently found that the fastest growth occurred at levels of 4.0 of auxins to 5.0 of cytokinins.

Other comparisons of callus growth on agar media

Many comparisons of callus growth on different media were made before attempting the precision of the factorial tests of growth regulator levels. They were made to gain broad information on which to base factorial tests. In these comparisons, portions of the same callus were inoculated on slants of several different media at the same time. Although other basal media were used in some experiments, for purposes of comparison, only those on Murashige and Skoog's medium will be discussed.

Five of the better growth regulator combinations and the estimated growth rates of callus upon them are shown in Table 5. The most rapid growth was achieved with mixture number 1. Estimated growth varied with explant source, age, firmness, and color of the different calluses measured. Very rapid growth, particularly on medium number 1, may have caused deficiencies to occur in the medium before transfers were made. Necrotic zones became apparent in the

TABLE 5.--CALLUS DIAMETER GROWTH ON VARIOUS GROWTH REGULATOR SUPPLEMENTS OF MURASHIGE AND SKOOG (1962) BASAL AGAR MEDIUM

Number	Medium supplements[a]	Estimated days to double callus diameter
1	1 mg/ℓ each IAA, NAA, 2,4-D, C, B, D, K, Ben, Z, + 2 mg/ℓ GA (No. 30, Table 2)	10-20
2	0.8 mg/ℓ each IAA, NAA, 2,4-D, IBA, C 1 mg/ℓ each B, D, K, Ben, BT (No. 38, Table 2)	12-24
3	2.5 mg/ℓ 2,4-D, 10% CM (No. 4, Table 2)	12-24
4	2.0 mg/ℓ 2,4-D & C, 2.5 mg/ℓ B & K	12-24
5	2.5 mg/ℓ 2,4-D, 5 mg/ℓ B (No. 27, Table 2)	15-30

[a] See list of abbreviations on page xiv.

calluses on this medium after about 3 weeks of growth.

The modifications of Table 5 were not the only media to cause rapid growth. Those shown were some that tended to be compatible with almost any source of callus. Others, such as a modification using 15 mg/ℓ of dimethylallyladenine, caused excellent growth of certain calluses, and seemed to actually inhibit growth of others. For example, the dimethylallyladenine medium produced excellent growth of one clone of root sucker tip callus, but a clone of seedling tip callus became necrotic whenever grown on the medium.

Another problem with many growth experiments was that conditioning during prior treatments seemed to influence growth of calluses on the current medium. This was particularly noticeable when material was transferred from liquid to agar media. Growth was almost invariably retarded when this was done.

Agar-grown calluses were transferred to other agar media and their growth was compared. They did not show an effect of the previous media on growth. The residual effect of relatively large amounts of benzyladenine was tested by comparing diameters of the three green and three white seedling tip callus explants after 6 weeks on the media shown (Table 6). The 5 mg/ℓ benzyladenine media permitted the most rapid growth in two of the original media treatments, but no relationship to original treatment was indicated by the data.

The growth of root sucker tip callus that had been grown in the sucrose/coconut milk factorial experiment (No. 7, Table 4) was compared after it was transferred from the sucrose coconut milk medium

TABLE 6.--AVERAGE DIAMETER OF CALLUSES ORIGINALLY 0.3 CM IN DIAMETER TRANSFERRED FROM VARIOUS BENZYLADENINE LEVELS TO OTHER MEDIA AND GROWN FOR 6 WEEKS

Former medium[a] supplements mg/ℓ B[b]	Growth medium[a] supplements			
	M[a]	10% CM	1 mg/ℓ B	5 mg/ℓ B
	Diameter			
10	0.3	0.4	0.5	0.3
15	0.3	0.5	0.4	1.0
20	0.4	0.5	0.4	0.4
25	0.5	0.4	0.7	1.1

[a] Murashige and Skoog basal medium.

[b] Benzyladenine.

to a slant containing basal medium with 15 mg/ℓ dimethylallyladenine. Again, in this experiment, no obvious carry-over effect from the previous media could be found in the diameter growth on the new media.

Media supplements that were found to be poor or very poor stimulators of koa callus growth on agar media are given (Table 7). Independently, at the levels shown, they had little beneficial effect on growth. The benzthiazoleyloxyacetic acid and the 2,4-D at 5 mg/ℓ caused rapid formation of necrotic zones in callus tissue. However, in combination, several of these supplements were found to be beneficial to growth.

A trial was made of Murashige and Skoog's basal medium modified by removing the ammonium nitrate from the formulation. This was done because good growth had been occurring in Schenk and Hildebrandt's

TABLE 7.--SUPPLEMENTS TO MURASHIGE AND SKOOG BASAL AGAR MEDIUM THAT DID NOT INCREASE CALLUS GROWTH

Supplements[a]	Levels used[b]
None	0
CM	5%, 10%
K	5, 10
Ben	10, 20, 30
B[c]	1, 5
2,4-D	5
Z	5
BT	5, 10

[a] See list of abbreviations on page xiv.

[b] mg/ℓ unless otherwise indicated.

[c] Benzyladenine contributes to organization, but not growth at low levels.

medium that contained no ammonium and it was felt that the ammonia might be inhibitory to koa callus. When this medium was supplemented with 2.5 mg/ℓ of 2,4-D and 10 percent coconut milk, seedling tip and stem callus grew at a slow rate. Also, many trials were made of Murashige and Skoog's basal media with 0.5 or 1 g/ℓ of myo-inositol rather than the usual of 0.1 g/ℓ. No difference was found in cultures grown with higher levels of this sugar alcohol.

As described previously, one clone of root sucker tip callus responded very well to basal medium modified with 15 mg/ℓ dimethylallyladenine. Even more rapid growth of this tissue was achieved when 0.2 mg/ℓ of 2,4-D was added. The same tissue also grew almost equally well on a mixture of 5 mg/ℓ of benzyladenine and 10 mg/ℓ dimethylallyladenine. Unfortunately, these media caused increased growth only with the one clone.

A list of all the media examined for growth promoting potential of callus and cell suspensions will be found in Appendix B. The media are listed with ratings of their effectiveness in promoting rapid growth of callus to occur.

Growth of callus and cell suspensions in liquid media

Koa callus growing in liquid media was found to behave in certain distinct patterns. It would remain in its original tightly bound form, break up into large aggregates which would become hard spheres, or, become soft, loosely-bound aggregates and individual cells. This last category is also called a cell suspension. Aggregation was found to be controllable to some degree by adding or removing coconut milk or, in some cultures, benzyladenine. The "addition or removal" was

usually done by moving the tissue from one medium to another via pipettes or forceps, rather than by adding the desired ingredients to a culture. This had the effect of applying a "selection" process to the subcultures. This selection may have had an influence on variation in results in any one medium.

Almost all information gathered on tissue growth in liquid cultures was qualitative. No measurements were made because the tissues were to be used in later experiments. Thus, the terms used to describe growth--rapid, normal, or slow--refer only to comparisons with other koa tissue under the same experimental conditions.

The most common medium used for both induction and growth of callus in liquid was Schenk and Hildebrandt's (1972) basal medium (Appendix A) modified with 0.2 mg/ℓ of 2,4-D, 2.0 mg/ℓ of p-chlorophenoxyacetic acid and 0.1 mg/ℓ of kinetin. Callus in this medium grew at what is termed a "normal" rate. At this normal rate callus would double in size, or volume, at about 1-month intervals. Most pieces of callus inoculated to this medium formed loosely bound cells which would become cell suspensions about 2 months after inoculation. If not removed from the original medium, after 4 to 5 months the tissue would develop roots or structures with both a root and a primordial shoot called embryoids.

Tissue in this medium would not form chloroplasts, but when tissue with chloroplasts was transferred to the medium the chloroplasts would be retained. The addition of 10 percent coconut milk to the medium caused the formation of chloroplasts and aggregation of cell suspensions into solid masses, but slowed growth

of the suspension.

All the growth regulator modifications made to Schenk and Hildebrandt's medium which increased callus and/or cell suspension growth are listed in Table 8. These media were, except in one instance, only used for growing tissue derived from seedling or root sucker tips. Seedling tip tissue almost always grew more rapidly than root sucker tip tissue in these media.

The best growth of cell suspensions occurred in medium 14 (Table 8) with seedling tip callus. These suspensions consisted of small clumps and individual cells rich in chloroplasts. Root sucker tip tissue grown in this medium and also in medium 15 retained its totipotency because it later formed adventitious shoots in other media.

The presence of kinetin along with benzyladenine inhibited growth in media 9 and 10. The absence of auxins in media 12 and 13 prevented growth. Medium 16, with a high content of cytokinins, caused good growth of callus, but the callus did not develop into suspensions.

A variety of cells and cell groups formed in suspension cultures. It was considered most desirable to obtain spherical, rapidly dividing cells (Fig. 14) because cells of this type have demonstrated totipotency (M. Mapes, personal communication). Small clumps were formed from which these spherical cells would break free. As mentioned, the addition of coconut milk to the medium would sometimes cause aggregation. When this occurred, some cell elongation accompanied by the development of starch grains (Fig. 15) would sometimes also take place. The development of starch grains did not

TABLE 8.--GROWTH REGULATOR SUPPLEMENTS OF SCHENK AND HILDEBRANDT (1972) BASAL MEDIUM USED IN LIQUID FORM AND THEIR EFFECT ON GROWTH OF CALLUS AND CELL SUSPENSIONS FROM VARIOUS SOURCES

Number	Supplements[a]	Source of tissue	Growth rate
1	0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ C, 0.1 mg/ℓ K	Seedling tips Seedling stems Root sucker tips	Normal Normal Slow
2	1 above + 10% CM	Seedling tips	Slow
3	0.2 mg/ℓ N	Root sucker stem	Slow
4	2.5 mg/ℓ N, 1.0 mg/ℓ K	Root sucker tip	Slow
5	0.2 mg/ℓ 2,4-D, 0.1 mg/ℓ K	Seedling & root sucker tips	Slow
6	2.0 mg/ℓ C, 0.1 mg/ℓ K	Seedling & root sucker tips	Slow
7	4.0 mg/ℓ C	Seedling & root sucker tips	Slow
8	1 above + 20 mg/ℓ Adn	Seedling & root sucker tips	Normal
9	1 above + 0.1 mg/ℓ B	Seedling & root sucker tips	Slow
10	1 above + 1.0 mg/ℓ B	Seedling & root sucker tips	Slow
11	0.1 mg/ℓ B	Seedling & root sucker tips	Slow
12	1.0 mg/ℓ B	Seedling & root sucker tips	Slow
13	2.0 mg/ℓ B	Seedling & root sucker tips	Slow
14	0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ C, 0.1 mg/ℓ B	Seedling tips Root sucker tip	Rapid Slow
15	0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ C, 1.0 mg/ℓ B	Seedling tip Root sucker tip	Normal Slow

TABLE 8. (Continued) GROWTH REGULATOR SUPPLEMENTS OF SCHENK AND HILDEBRANDT (1972) BASAL MEDIUM USED IN LIQUID FORM AND THEIR EFFECT ON GROWTH OF CALLUS AND CELL SUSPENSIONS FROM VARIOUS SOURCES

Number	Supplements[a]	Source of tissue	Growth rate
16	0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ C, 5.0 mg/ℓ B, 5.0 mg/ℓ K	Seedling tip	Normal
17	0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ C, 10.0 mg/ℓ B, 5.0 mg/ℓ K	Seedling tip	Slow

[a] Abbreviations for growth regulators on page xiv.

serve as indicator of shoot development as had been found by Murashige (1974b). Ultimately, in media containing auxin, aggregates in the cell suspenions would form embryo-like structures (Fig. 16).

The other basal medium used in liquid culture experiments was that of Murashige and Skoog (1962). Most growth regulator modifications to this medium were similar to those used in agar media (Table 4). However, in liquid culture, the tissue is totally surrounded by the media and thus more easily damaged by high concentrations of growth regulators. Therefore, some of the growth regulators were used in reduced amounts.

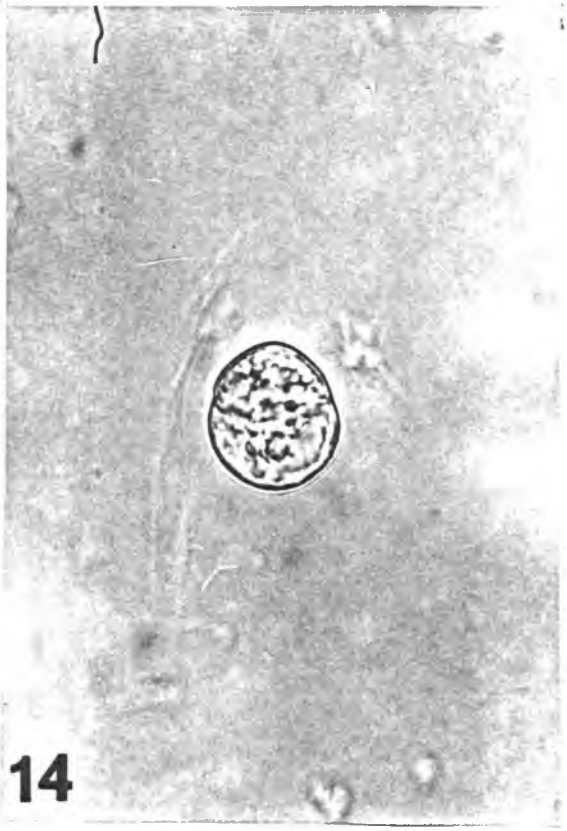
It was found that existing cell suspensions would survive for a month or more on unsupplemented basal medium. The addition of 5 or 10 percent coconut milk to Murashige and Skoog's basal medium caused aggregation or rapid divisions of some cells, and in some instances (Fig. 17) caused the initial stages of organization of the callus tissue back into shoots to occur. This was indicated by groups of

Figure 14. Densely cytoplasmic single cell from suspension culture culture grown in Schenk and Hildebrandt's basal medium supplemented with 2,4 dichlorophenoxyacetic acid and kinetin.

Figure 15. Cell aggregates with starch grains formed after 10 percent coconut milk was added to modified Schenk and Hildebrandt's basal medium.

Figure 16. An embryoid forming with central vascular system connecting a dome with leaf primordia-like attachments and a root primordia at the base which is high in anthocyanin.

Figure 17. A meristematic zone (left center) formed in liquid Murashige and Skoog's basal medium with 10 percent coconut milk in a root sucker tip callus suspension. Note the prominent nuclei and low vacuolation of these cells.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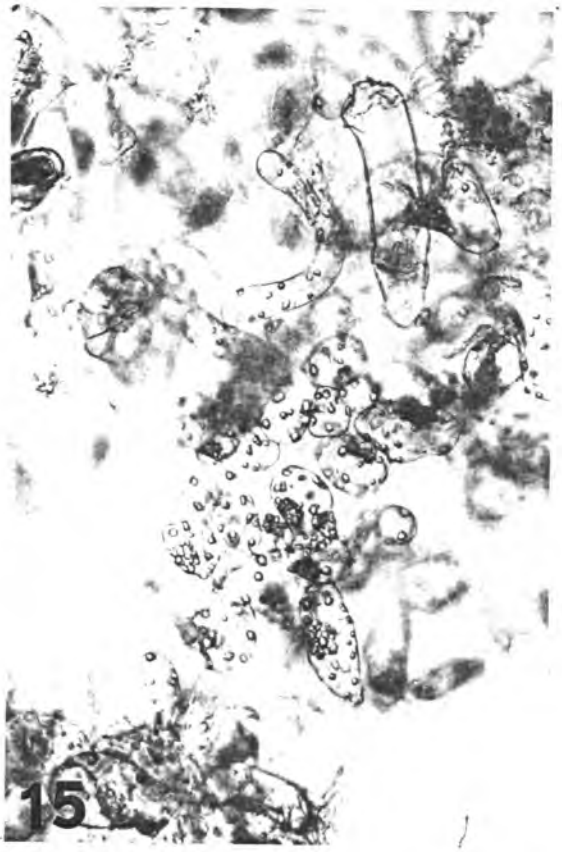
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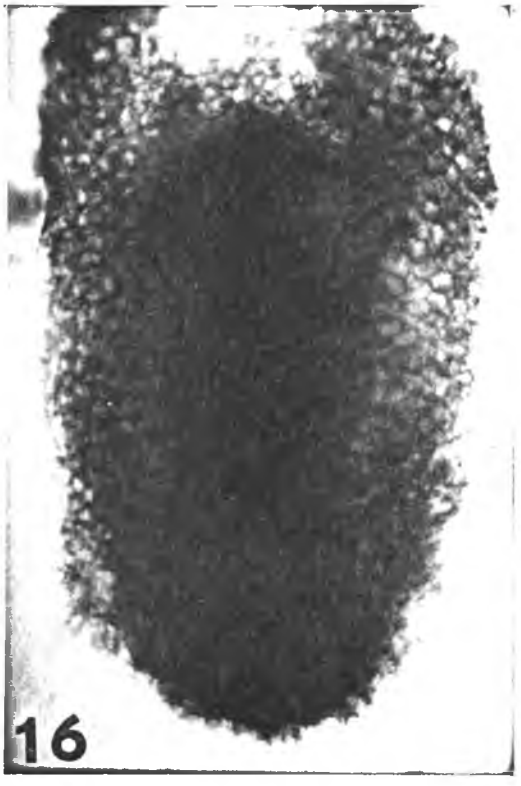
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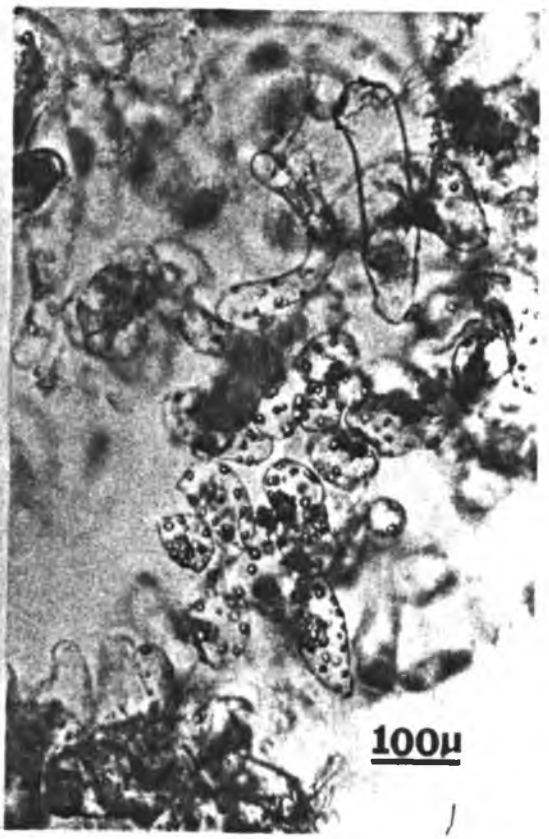
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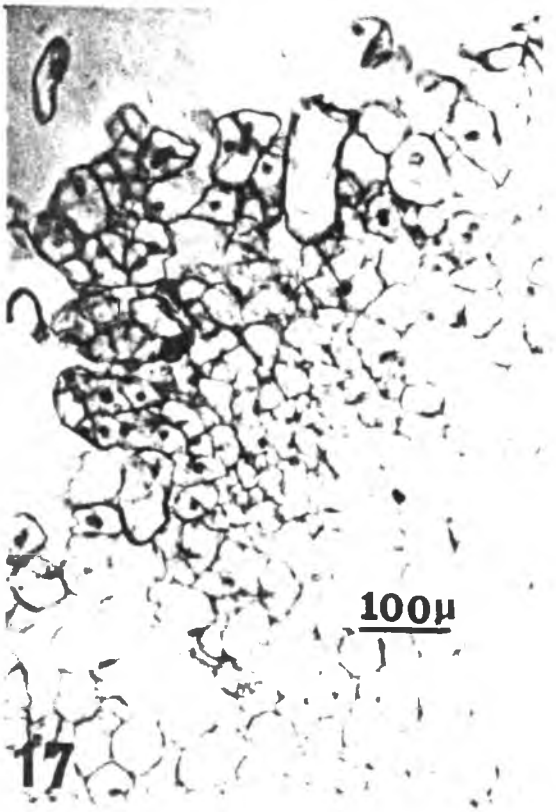
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17

small, rapidly dividing cells with reduced vacuolation and prominent nuclei. Growth of tissue in media supplemented with coconut milk was, however, usually slow.

One factor that could not be examined in agar media was the effect of media pH on tissue growth. By chance, it was noticed that the pH of a liquid medium had been changed by autoclaving from the recommended 5.7 it had been adjusted to, to 4.8. This led to a series of tests aimed at determining the effect of medium pH on koa callus growth. These tests were made using Murashige and Skoog's basal medium supplemented with 2.5 mg/ℓ of 2,4-D and 10 percent coconut milk.

The effects of heat and cold on pH change, were investigated by making up aliquots of media at seven pH levels, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. One-half of each aliquot was frozen overnight and the other one-half stored in a refrigerator. The pH of the frozen samples was measured after thawing at the same time as that of the refrigerated samples. Only a slight pH change was noted in the frozen samples (Table 9), and almost none occurred in the cooled samples. The media were then placed in 50 ml erlenmeyer flasks and autoclaved at 121^{o} C for 20 minutes. After autoclaving, the media of two flasks at each pH level were measured again (Table 9).

The large reduction in pH of the autoclaved media at pH 5 and above indicated that all cultures might have been grown on media at much less than the recommended acidity of pH 5.7. Tests of other media in stock, and more that were freshly made, indicated similar reductions caused by autoclaving. One medium (Number 1 of Table 5),

TABLE 9.--EFFECTS OF REFRIGERATION, FREEZING, AND HEATING ON pH OF 2.5 mg/ℓ 2,4-D, 10 PERCENT COCONUT MILK MODIFIED MURASHIGE AND SKOOG BASAL MEDIUM

Before freezing or refrigeration	After refrigeration	After freezing and thawing	After autoclaving
4.00	4.01	4.04	4.04
4.50	4.50	4.52	4.46
5.00	4.99	5.01	4.89
5.50	5.50	5.51	5.17
6.00	6.01	6.02	5.44
6.50	6.50	6.51	5.71
7.00	7.00	6.99	6.00

when made at pH 6.00 was reduced to 5.58 after one autoclaving and to 4.61 after a second autoclaving. This degree of change was borne out by numerous other tests of other media, so from that time onwards, all media were adjusted to pH 6.0 before autoclaving in the hope that less influence of acidity would result.

The remaining flasks from this experiment were inoculated with root sucker and seedling tip callus from liquid media to test the effects of the different pH levels. After 6 weeks of growth, the fastest growth of both seedling and root sucker tip tissue had taken place in the media originally at pH 5.5. All cultures contained abundant chloroplasts, and greenness generally increased with decreasing pH. After 11 weeks, the most vigorous, "healthy" appearing cultures were in the pH 6.0 treatments.

The pH of all treatments was measured at the end of 11 weeks (Table 10) and had changed markedly in use. The tissue in the cultures had modified the media pH in all treatments. The initial shock of placement in a very acid medium may have set back the growth of the callus in media having a pH of 5.0 and lower.

TABLE 10.--CHANGES IN pH OF MURASHIGE AND SKOOG BASAL MEDIUM WITH 2,4-D AND COCONUT MILK AFTER AUTOCLAVING AND USE AS A CULTURE MEDIUM FOR 11 WEEKS

Nominal pH	pH after autoclaving	pH after use	
		Seedling tip	Sucker tip
4.0	4.04	5.61	5.23
4.5	4.46	5.37	5.13
5.0	4.89	5.31	5.51
5.5	5.17	5.95	5.51
6.0	5.44	5.60	5.24
6.5	5.71	5.43	5.75
7.0	6.00	5.66	5.70

Many other supplements for liquid Murashige and Skoog's basal medium were used and many were found to cause a rapid or at least normal rate of growth (Table 11). Not all the liquid media used for increasing tissue growth are shown, only those that were more successful. A complete list of media tried will be found in Appendix B.

The most rapid growth of tissue in liquid occurred in medium 1 (Table 11) regardless of the source of the tissue. Similar growth

TABLE 11.--GROWTH REGULATOR SUPPLEMENTS OF MURASHIGE AND SKOOG (1962) BASAL MEDIUM USED IN LIQUID FORM AND THEIR EFFECT ON GROWTH OF CALLUS AND CELL SUSPENSIONS FROM VARIOUS SOURCES

Number	Supplements[a]	Source of tissue	Growth rate
1	1 mg/ℓ each IAA, NAA, 2,4-D, C, B, D, K, Ben, Z, and 2 mg/ℓ GA	Any	Rapid
2	1 above without GA	Seedling & root sucker tips	Normal
3	1 above with BT instead of Ben	Root sucker tips Root sphaeroblast	Normal
4	2 above with BT instead of Ben	Root sucker tip	Normal
5	1 above with BT instead of Z and with IBA	Seedling tip Root sucker tip	Normal
6	5 above with 0.8 mg/ℓ each auxin	Any	Rapid
7	5 above with 0.6 mg/ℓ each auxin	Any	Rapid
8	15 mg/ℓ D	Root sucker tip	Rapid
9	10 mg/ℓ D	Root sucker tip	Normal
10	15 mg/ℓ D, 0.2 mg/ℓ 2,4-D	Root sucker tip	Rapid
11	5 mg/ℓ B, 10 mg/ℓ D	Root sucker tip	Rapid
12	2.5 mg/ℓ 2,4-D, 10% CM	Seedling tip, stem Root sucker tip	Rapid Normal
13	2.5 mg/ℓ 2,4-D, 1% CM	Seedling tip, stem	Normal
14	1.25 mg/ℓ 2,4-D, 1% CM	Seedling tip, stem	Normal
15	2 mg/ℓ each IAA, NAA, 2.5 mg/ℓ each B, D	Seedling tip Root sucker tip	Normal
16	2 mg/ℓ each C, NAA, 2.5 mg/ℓ each B, D	Seedling tip Root sucker tip	Normal

TABLE 11. (Continued) GROWTH REGULATOR SUPPLEMENTS OF MURASHIGE AND SKOOG (1962) BASAL MEDIUM USED IN LIQUID FORM AND THEIR EFFECT ON GROWTH OF CALLUS AND CELL SUSPENSIONS FROM VARIOUS SOURCES

Number	Supplements[a]	Source of tissue	Growth rate
17	2 mg/ℓ each B, D, K, Ben, BT, GA	Seedling tip Root sucker tip	Slow
18	17 above with 10% CM	Seedling & root sucker tip	Normal
19	10% CM	Seedling & root sucker tip	Slow
20	5% CM	Seedling & root sucker tip	Slow

[a] List of abbreviations are on page xiv.

occurred with media 6 and 7. The rapid growth in these media quickly created nutrient deficiencies and frequent subculturing was essential to maintain the growth rate. Cultures in these media remained white or produced a very small number of chloroplasts. They separated quickly into cell suspensions.

Liquid media in which root sucker tip callus grew rapidly were numbers 8, 10, and 11. Despite the rapid growth, cell suspensions were maintained in these media for over 3 months before deficiency symptoms began to appear. Medium 10 caused particularly rapid growth, forming "thick" suspensions from explants of only a few thousand cells in 6 to 8 weeks. These media also induced adventitious shoots to form in the suspended tissue.

Medium 12 also caused rapid growth of seedling tip and stem

tissue, but the tissue grown in this medium did not show regeneration ability in any subsequent subcultures. Media 13 and 14 caused cell suspensions to form quickly from callus, but the suspensions then produced roots which indicated a loss of capacity to develop shoots. The first embryoids to form, however, formed in medium 13.

Media 15 and 16 were two of many that were used in a search for the growth regulators that caused most of the activity in medium 1. The rate of growth achieved with medium 1 was never equalled with any of the 4 mg/ℓ of auxin, 5 mg/ℓ of cytokinin combinations, such as media 15 and 16, that were tried. But medium 15 did grow tissue of both seedling tips and root sucker tips quite well. It also induced adventitious shoot development in one root sucker tip cell suspension culture.

Media 17 and 18 were attempts to induce shoots, but proved to be suited somewhat better for the growth of callus. However, three adventitious shoots did form on media 17, so the high level of cytokinins had the intended effect to a small degree. These media did not interfere with the regenerative capacity of the tissues inoculated to them. Subcultures later produced shoots.

Discussion

Koa callus requires both auxin and cytokinin for good growth as was made clear from the results of many experiments. In all experiments in which only a cytokinin or group of cytokinins were used to supplement the basal medium, except for one, growth of callus was much reduced as compared to growth on media containing both an auxin and cytokinin. This was in keeping with the findings of many others

(Skoog and Miller 1957, White 1963, Steward 1970).

Callus from seedling stems outgrew callus from any other source when growing as white tissue. When grown on media containing 5 or 10 percent coconut milk or 5 mg/ℓ benzyladenine, some seedling stem tissue would form chloroplasts, but this tissue, when compared with the original chlorophyll-free callus on the same medium, grew much more slowly. The presence of abundant chloroplasts was associated with slower growth in all the comparisons made. Chlorophyll formation in the callus tissue seemed to result in a lowered energy available for cell division and enlargement.

Although seedling stem callus grew most rapidly, most experimentation on accelerating callus growth was done with seedling and root sucker tip callus and cell suspensions because tissue from tips had been found in other experiments to retain the capacity to regenerate shoots. Stem tissue did not appear to have this capability, nor did tissue from most other sources.

Koa callus grew well when coconut milk was used in place of a cytokinin only when an auxin was also present in the medium. In the absence of auxin coconut milk alone did not supply the stimulation required for rapid growth except in one instance, which could not be repeated. The effects of coconut milk on tissue condition in addition to increased chlorophyll formation, were aggregation of cell suspension cultures, development of starch grains in cell suspensions, and reduced vacuolation, enlarged nuclei, and multiple divisions in liquid cultures. All such changes are consumptive of energy that would otherwise be available for more rapid growth of the tissue which

suggests the reason why coconut milk was not as supportive of rapid growth as were cytokinins.

The reason for a mixture of growth regulators to be more reactive than individual regulators is unknown. Possibly, different molecules of auxin become attached to different stereospecific sites, stimulate different types of RNA to be produced and thus different enzymes, different anti-auxin formation, or perhaps release different already available growth limiting substances. The net result would be augmented auxin reactions--cell wall softening, increased turgor, increased RNA synthesis. Similarly, different cytokinin molecules might attach to different RNA groups, as in fact, they have been found to do (Hall et al. 1967, Skoog and Armstrong 1970, Fox and Chen 1967, Deleuze et al. 1972, Parker et al. 1972. Zeatin, dimethylallyladenine, and benzyladenine have been found to attach to specific RNA structures and zeatin and benzyladenine to actually become incorporated in stable glucosylated purines. Therefore, it is quite reasonable to expect different reactions from different cytokinins and, perhaps, as is the case with koa, an enhancement of reaction by providing five different molecules of cytokinins. The role of gibberellic acid is not at all clear, but numerous trials indicated an improvement in callus growth when it was present. Nitch and Nitch (1963) found gibberellic acid to be rapidly converted to a stable glucoside, which others (Lang 1970, Loveys and Wareing 1971) have speculated may become available in active form through stimulation of additional gibberellic acid or other gibberellins.

An important factor in koa tissue culture may be that the tissue

does not seem to form growth inhibitory phenolics as do tissues of many other woody plants. Phenolics include numerous inhibitors which are believed to function by interference with oxidative phosphorylation and the enhancement of IAA oxidase (Pridham 1965). However, some (Nitch and Nitch 1962) can inhibit IAA oxidase at low concentrations and actually stimulate growth. Because of the low tendency to form phenolics, or to "brown", koa callus and cell suspensions were frequently held for as long as 4 months without transfer to fresh medium. This allowed time for the tissue to adjust to the medium, to show measurable change in size and condition, and in some instances, to differentiate.

It was quite unusual that the tissue would often respond well to the same concentrations of growth regulators in both agar and liquid cultures. In most tissue culture work, growth regulator concentrations of 0.1 to 0.2 mg/ℓ, and even lower are usually used in liquid cultures. Although koa tissue grew well at low growth regulator levels in Schenk and Hildebrandt's liquid media, it grew even better at very high levels in Murashige and Skoog's liquid media. Yet, when tested by factorial experiment on agar, the best growth usually occurred at less than the maximum level of growth regulators, indicating an inhibitory effect that was not seen in comparing growth in liquid cultures of high and low concentrations.

There was a noticeable effect of age of tissue on growth. Newly developed callus would always grow more rapidly than older callus. However, because of the selection process inherent in subculturing small pieces of callus, or pipetting cell clusters, a large variation

in growth rate developed among the subcultures which made the estimation of the effect of culture age on growth rate difficult. Also, movement from liquid to agar or agar to liquid inhibited growth.

Much evidence has been shown for the "conditioning" of medium by the tissue grown in it (Steward et al. 1975). Both the medium and the tissue become "attuned" to each other. A "conditioned" medium can be pipetted into a fresh medium and cause the fresh medium to induce freshly inoculated tissue to become similar to tissue grown in the "conditioned" medium. Evidence of medium "conditioning" occurring in koa tissue culture was provided by the uniformity in pH after use in culture of liquid media that had varied in pH prior to inoculation. As indicated in Table 10, the pH of the medium when inoculated had little relationship to the pH of the medium after use for 11 weeks. In all instances the tissue being cultured ameliorated the media pH towards the level of acidity at which nutrients would be more readily available. The medium is affected by the tissue while the tissue is affected by the medium.

The finding that autoclaving has a large effect on media pH seems to have been largely overlooked by most tissue culturists. Vacin and Went (1949) did some work in this area in developing their orchid medium. But their medium was much more acid than those used for koa culture and may have been less affected by autoclaving.

Conclusions

The results have indicated that numerous kinds and levels of auxins and cytokinins can be added to either Schenk and Hildebrandt's or Murashige and Skoog's basal media to obtain a medium that will cause rapid growth of koa callus, or cell suspension tissue. There are so many choices that it is difficult to select just one system as the best.

If one system were to be selected for increasing tissue growth, it would have to be Murashige and Skoog's basal medium in liquid form supplemented with 1 mg/ℓ each of indoleacetic acid, napthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, p-chlorophenoxyacetic acid, benzyladenine, dimethylallyladenine, kinetin, benzylaminobenzimidazole, zeatin, and 2 mg/ℓ of gibberellic acid. With frequent subculturing every 2 to 3 weeks, larger amounts of tissue with a capacity to regenerate shoots could be grown by this system than any of the others investigated. The explant sources resulting in the greatest totipotency were shoot tips. The best system for growing the tissue rapidly was in erlenmeyer flasks on an orbital shaker at 100 rpm under 30 or more $\mu E\ m^{-2}sec^{-1}$ of light.

Actually, this medium and supplements whether in liquid or solid form were the only ones that were found to produce totipotent callus from field-collected root sucker tips. With selection, it was found possible to further increase root sucker tip tissue in basal media containing 0.2 mg/ℓ 2,4-D and 15 mg/ℓ dimethylallyladenine, or just 15 mg/ℓ dimethylallyladenine, but this was only successful for one clone.

Several alternative methods could be used to increase seedling

tip callus. The simplest and best media would probably be liquid Schenk and Hildebrandt's basal medium supplemented with 0.2 mg/ℓ 2,4-D, 2 mg/ℓ p-chlorophenoxyacetic acid and 0.1 mg/ℓ kinetin. This medium caused callus induction and normal growth of seedling tip callus and cell suspensions that later exhibited totipotency. However, more rapid growth was achieved with the same basal medium supplemented with benzyladenine in place of the kinetin with no loss in totipotency. Still more rapid growth of seedling tip callus was obtained with the mixture of four auxins and five cytokinins discussed above.

Growth regulators that did not cause normal callus growth of koa tissue when used singly with Murashige and Skoog's basal medium were coconut milk at 5 or 10 percent, kinetin at 5 and 10 mg/ℓ, benzylaminobenzimidazole, at 10 to 30 mg/P, benzyladenine at 1-5 mg/ℓ, 2,4-D at 5 mg/ℓ, zeatin at 5 mg/ℓ and benzthiazolyloxyacetic acid at 5 to 10 mg/ℓ. These require supplementation with other growth regulators to achieve improved growth.

To obtain supplemented Murashige and Skoog's basal media with a pH of approximately 5.7 which will allow rapid growth of koa tissue, media should be adjusted to pH 6.0 before autoclaving. If this is not done, the initial acidity of the medium may set back the growth of tissue inoculated to it by reducing nutrient availability.

CHAPTER 3

EMBRYOGENESIS, ORGANOGENESIS, AND DEVELOPMENT OF COMPLETE ROOTED TREES FROM CALLUS AND CELL SUSPENSION CULTURES OF *ACACIA KOA* GRAY

Introduction

The regeneration of plants from unorganized callus tissue or cell suspensions has been worked out empirically for numerous species. The complexity of methods ranges from the simple expedient of leaving the tissue in the same medium for many weeks with such easy-to-propagate plants as orchids to rather elaborate control of light and growth regulators by making several media changes, or tissue transfers.

Cloning, or vegetative propagation of *Acacia koa* trees by callus and cell suspension techniques required that the essentially undifferentiated tissue be induced to differentiate, or organize, into structures that would then form whole plants. These whole rooted plants then would have to be removed from aseptic, in vitro conditions and grown under normal conditions outdoors. Successes in accomplishing these events in tissue culture of forest trees are still quite rare.

The approach used with koa tissue was to seek out the proper kinds and balance of growth regulators that would cause cells in the callus or cell suspension cultures to organize into meristemoids, which were then induced to form roots or shoots. If the tissue first formed roots, growth regulators were generally afterwards withheld so that cytokinins formed in the roots might produce shoots. If the tissue formed shoots, they were caused to elongate and leaf out by providing small amounts of cytokinins. Then the shoots were caused to

root by adding auxins. Once roots had grown, the plantlets had to be removed from the aseptic culture. Numerous experiments were carried out to develop a technique for aftercare by which the plantlets could be grown as autotrophic entities.

The experiments resulted in many different types of organization. Certain tissues formed roots, others embryoids, still others formed shoots, some of which proliferated. One clone of seedling tip callus produced numerous complete rooted trees that have now been planted in the forest and are growing as normal trees. One event, the formation of shoots in cell suspension cultures, is at the present time unique in the field of forest tree tissue culture.

Literature Review

The requirements for differentiation or organogenesis to occur in callus tissue and cell suspensions are often complicated and diverse among different species and different types of tissue (Murashige 1974b). The physiologic age and the season of collection of the original explant tissue and the age and condition of the culture derived from it all play roles (Murashige 1974b, Raju and Mann 1970) in determining the ability of the culture to regenerate. The basal medium can be influential as well. The phosphate concentration, for example, can be increased to weaken the root forming potential of auxins and augment shoot formation (Reinert 1973). Some researchers (Reinert 1973, Durzan and Lopushanski 1975) have shown improved organogenesis with the addition of organic nitrogen to the media.

The kind and amount of growth regulators required for differentiation differ greatly from tissue to tissue. Mehra and Mehra

(1974) obtained shoots of almond leaf callus most frequently using 5 mg/ℓ napthaleneacetic acid and 1 mg/ℓ kinetin. Herman and Haas (1975) obtained shoots from coffee callus using 0.1 mg/ℓ each of 2,4-D and kinetin. Durzan and Lopushanski (1975) obtained shoots from elm callus by removing all auxin and cytokinin, while Winton (1970) obtained them from aspen callus using no auxin, but 0.15 mg/ℓ benzyladenine. This gives some indication of the diverse requirements of callus of just a few tree species.

The laboratory requirements, particularly light quality and quantity, often play a large role in differentiation. Siebert, Weatherbee, and Job (1975) found that a low level (0.024 $mwcm^{-2}$) of purple light (371 nm) caused shoot formation in tobacco callus, while just slightly more light (0.15 $mwcm^{-2}$) of the same wave length inhibited it. Kakade and O'Connor (1977) found that Douglas-fir embryo cultures formed adventitious buds at a rate five times that of any other light quantity or quality when grown at 0.42 $mwcm^{-2}$ of 660 nm light--very different than tobacco callus. Light at 371 nm had no bud forming effect on Douglas-fir tissue. These data serve to indicate that the reactions to light are quite distinctive among different species of callus tissue. Winton (1974), for example, at times found shoot formation to occur much more frequently in aspen callus that had been grown in the dark than in the light.

Other environmental controls on differentiation of callus and cell suspensions have been examined to varying degrees. Most tissue culturists (Steward et al. 1952, Street 1973b, Winton 1974) use temperatures ranging from 21^{o} to 27^{o} C. Some (Mehra and Mehra 1974,

Murashige 1974b) vary daytime and nighttime temperature and "day" length. Others (Steward et al. 1952) use constant light and temperature. Some (Steward et al. 1952, Steward 1970) attribute excellent organization in part to the use of specialized glassware and clinostats. Others (Winton 1970, Staritsky 1970) have obtained good differentiation using more primitive techniques and equipment. It is not clear from the literature in this rather new field of tissue culture what environmental requirements are really essential for organization to occur, because the techniques and material used have varied so much.

Skoog and Miller (1957) discovered that auxin and cytokinin interact in root and shoot formation which provided the key to organogenesis and propagation by tissue culture. Although other tissues have been found to react quite differently than the tobacco pith callus they worked with, the basic reactions hold generally for angiosperm tree tissue (Winton and Huhtinen 1976). These reactions, as suggested by Torrey (1966), begin with the formation of a "meristemoid," a tight group of two or several very small cells with prominent nuclei and little or no vacuolation. According to Torrey (1966), these cells have the capacity to organize into roots, shoots, or embryos, depending on the stimulation received. The nature of this stimulation is not clearly understood (Reinert 1973), but in almost all tissue culture work it has been found to be associated with the level of auxin or cytokinin supplied. If the level of auxin in the tissue is increased the meristemoid will become a root meristem. If cytokinin is increased it will become a shoot meristem. Bonnett and

Torrey (1966) have shown that the process can go in either direction. Steward et al. (1964) were the first to identify the initial stages of meristemoids becoming embryoids.

Roots develop readily from most cultured tissue under the influence of auxin (Murashige 1974b). However, embryoids and shoots are developed with less frequency and under much more specialized conditions (Durzan and Campbell 1974). As has been indicated, a certain quality and quantity of light may be required, but in addition the kind and the amount of cytokinin required to stimulate shoot formation may vary greatly (Mehra and Mehra 1974). The effects of growth regulators upon the meristemoid and developing shoot change as morphogenesis proceeds (Steward et al. 1969), and changes in the growth regulators must be made. This has been called sequential treatment (Steward et al. 1967). In shoots developing from the top of a piece of callus tissue, or within an aggregated mass of cells in suspension, the surrounding cells may "buffer" or "ameliorate" the action of the growth regulators upon the shoots, reducing the precision of sequencing growth regulators required for continued morphogenesis (Steward et al. 1969). In free cell suspensions, or where shoots develop on the surface of cell aggregates in liquid media, there are few or no cell membranes between the developing tissue and the medium to provide "amelioration," so sequencing of growth regulators is more critical (Steward et al. 1967).

In some tissue culture propagation, meristemoids have become shoots and developed into whole rooted plantlets without any changes to the growing medium (Murashige 1974b), in many others (Mehra and

Mehra 1974, Durzan and Campbell 1974, Murashige 1974b), sequential steps have been required. Most commonly (Murashige 1974b), the level of cytokinin is reduced or eliminated once shoots have started to develop. Sequential transfer of tissue back and forth to low and higher levels of cytokinins (Skolmen and Mapes 1976) was used to obtain gradual rather than abrupt change.

Almost all tissue culturists working with woody plants report difficulty in rooting tissue culture-produced shoots (Durzan and Campbell 1974, Cheng 1975, Pierik 1975, Winton and Huhtinen 1976, Brown and Sommer 1977). Successes in rooting shoots from tree tissues (Winton 1970, Mehra and Mehra 1974, Durzan and Lopushanski 1975) have usually been achieved by placing the shoot on a medium containing an auxin.

Many woody plant tissues produce high levels of phenolics in culture and numerous inhibitors of rooting may thus be formed (Pridham 1965). It has been shown that a number of phenolics interfere with oxidative phosphorylation and enhance indoleacetic acid oxidase formation, both of which could readily reduce root formation. Changes in RNA brought about by incorporation of cytokinins (Hall et al. 1967, Fox and Chen 1967), may also play a role, although there is no evidence for any difference between herbaceous and woody plants in this incorporation.

Brown and Sommer (1977) state that aftercare of aseptically cultured woody plants requires a large amount of research. "Aftercare" is removal of rooted plantlets from aseptic culture flasks and their conditioning to become autotrophic entities. With most

tissue culture propagated plants, normal nursery practice seems to be sufficient (Murashige 1974b). The plantlets are potted, placed under mist and after a time hardened off for field planting. Brown and Sommer (1977) have experienced poor success with such techniques applied to tissue culture propagated pines. They believe something more is needed. Wochok and El-Nil (1977) report similar findings with tissue culture-propagated Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]. Only about 10 percent of their shoots root and only a portion of the rooted plantlets survive the period of aftercare.

So far, only one paper has appeared that compares the variation within and between clones of tissue cultured trees, using the actual trees produced rather than cells, callus, or plantlets. Lester and Berbee (1977) showed that there was large variation in height, number of branches, and leaf form among six clones of Euromericana hybrid poplar produced by tissue culture. Root tip chromosome counts were also variable. They suggested that tissue culture-propagated plants will require clonal testing because they do not necessarily breed true. Sunderland (1973) shows considerable evidence that questions whether perpetuation of genotypes can be achieved by tissue culture.

Materials and Methods

Callus and cell suspensions of *Acacia koa* tissue from various sources were grown on agar-gelled or in liquid nutrient media containing all the necessary constituents to support growth. Organogenesis was caused to occur by subculturing the tissues to media containing different amounts or kinds of growth regulators. Growth and development of the newly formed organs were similarly controlled

until rooted plantlets had been obtained. These plantlets were then removed from aseptic conditions, rooted more completely in a liquid medium, and then potted. After several months they were planted in the field. That was the general procedure; the details of the methods follow.

Two basal nutrient media, Murashige and Skoog (1962) and Schenk and Hildebrandt (1972), were used in embryogenesis and organogenesis. The formulations of these two media appear in Appendix A. Schenk and Hildebrandt's medium was used only as a liquid and only for growth of cell suspensions and their organization into embryoids. Murashige and Skoog's medium was used for all other organogenesis. Another basal medium, Hoagland's solution (Hoagland and Arnon 1950) was used at full strength in liquid form as a rooting medium for plantlets removed from aseptic culture flasks.

Growth regulators of various kinds were added to the basal media in various combinations and amounts to cause differentiation to occur. The growth regulators used appear in the list of abbreviations on page xiv. The levels and combinations in which they were used appear in the results. In general, high levels of auxins and low levels of cytokinins or coconut milk, were used to cause the formation in callus and cell suspensions of roots and embryoids. High levels of cytokinins with low levels of auxins were used to cause shoots to form. Low levels of cytokinins were used to promote shoot elongation and leaf development and low levels of the auxin indolebutyric acid were used to root leafy shoots.

Rooted plantlets were grown after removal from aseptic media for

approximately 1 month in flasks of Hoagland's solution. During this time they were kept covered with polyethylene bags to reduce transpiration. They were then potted in a peat moss-vermiculite-perlite potting medium and grown under strong illumination for an additional month, for the first week of which they were held in polyethylene bags. Then they were transplanted to 1 quart plastic bags and grown in a greenhouse for about 3 months after which they were planted out in the forest. This process was worked out by experimentation explained under results.

Organogenesis of shoots took place on and within calluses growing on test tube slants of gelled media, in agitated erlenmeyer flasks of liquid media, and in slowly rotated t-tubes and nipple flasks (Steward and Shantz 1956, Street 1973b p. 61, 62) of liquid media. These flasks and slants were maintained on continuously lighted shelves or apparatus in one or the other of two culture rooms which were kept at a constant 25^{o} C. Agar gells were made using 8 g/ℓ of Bacto agar.

The photon flux density of photosynthetically active radiation (400 to 700 nm) used in embryogenesis, organogenesis and shoot growth was measured with a Lambda LI-170 quantum sensor manufactured by the Lambda Instruments Corporation. The data were reported as micro einsteins per square meter per second ($\mu E\ m^{-2}sec^{-1}$).

Two types of fluorescent lamps were used, "Cool-white" and "Gro-lux" (Westinghouse). Slants of callus, and flasks of shoots were kept on shelves approximately 30 cm distant from Cool-white or Gro-lux lamps. Agitated and rotated flasks were illuminated only with Cool-white lamps. The Gro-lux lamps provided nearly equal proportions of

light in the ranges 380 to 480, 480 to 630, and 630 to 700 nm, while the Cool-white lamps provided 60 percent of their light in the 480 to 630 nm range (Klein and Klein 1970). Studies with tobacco (Siebert et al. 1975) and Douglas-fir (Kakade and O'Connor 1977) have shown that shoot differentiation is influenced most strongly at the blue and red ends of the spectrum.

Cultures were observed daily for signs of organogenesis. A new shoot would be excised and transferred to a new medium as soon as leaf primordia had formed. It would then be transferred to new media at approximately monthly intervals until placed on a rooting medium. It would be left on the rooting medium for 3 months and if unrooted, returned to a medium without growth regulators. Rooted plantlets were grown on the rooting medium or in an auxin-free medium until the roots equalled the tops in size, then they were placed in Hoagland's solution in erlenmeyer flasks as has been mentioned.

The experiments that were undertaken have already been indicated by the foregoing, which has outlined some of those procedures, among the many tested, that were found to be most successful in causing callus and cell suspensions to organize into shoots and shoots to grow into trees. They consisted of completely randomized and randomized block factorial experiments in which treatments consisted of sources of tissue, media supplements, and laboratory conditions required to obtain differentiation of unorganized callus and cell suspensions. After differentiation had occurred, the experiments evaluated treatments aimed at growth and development of the organized tissue back into trees. Most of these experiments were trials of several

levels of one or more growth regulators upon callus or cell tissue of a particular type and condition to determine the most effective levels and kinds of growth regulators to use.

Results

Organization of Roots and Embryogenesis

Acacia koa tissue, just like tissue of other species (Murashige 1974b), was found to organize into roots quite readily. Roots were formed in both agar-gelled and liquid media from embryo, seedling stem, seedling tip, and root sucker tip callus, and cell suspensions (Table 12). Seedling stem tissue was the tissue most readily induced to form roots.

In most instances of root organogenesis, a relatively high level of auxin was included in the medium together with a relatively low level of coconut milk, or cytokinin (Table 12). In four cultures, root formation occurred in the absence of coconut milk or cytokinin, but in all instances, the previous medium had contained the missing substance. In the last two cultures in Table 12, roots developed when the tissue was transferred from a high cytokinin medium to unsupplemented low-salt Knudson's medium. This reaction only occurred with cell suspensions of root sucker tip tissue.

Embryogenesis occurred only in liquid media containing growth regulators similar to those that induced roots. There was, however, one important difference. The levels of auxins were much lower and more nearly equal to the levels of cytokinins or coconut milk in the embryoid forming media systems. Three systems were found that resulted in embryoid formation from cell suspension cultures. One,

TABLE 12

ROOT ORGANOGENESIS FROM CALLUS AND CELL SUSPENSIONS BY SOURCE OF TISSUE AND MEDIA

Original explant	Basal media[a]	Supplements[b]	Previous supplements[b]
Embryo	2ℓ	2,4-D 0.2, C 2.0, K 1.0	2,4-D 0.2, C 2.0, K 1.0
Seedling stem	1	2,4-D 1.25 CM 1%	2,4-D 2.5 CM 10%
Seedling stem	1	2,4-D 5.0 CM 1%	2,4-D 2.5 CM 10%
Seedling stem	1ℓ	2,4-D 2.5 CM 1%	CM 10%
Seedling stem	1	2,4-D 2.5 CM 0.5%	2,4-D 2.5 CM 10%
Seedling stem	1	C 5.0	2,4-D 2.5 CM 10%
Seedling stem	1	2,4-D 1.6	2,4-D 2.5 CM 10%
Seedling stem	1	2,4-D 0.8 CM 2.5%	2,4-D 2.5 CM 10%
Seedling stem	1	2,4-D 0.4 CM 5%	2,4-D 2.5 CM 10%
Seedling stem	2ℓ	NAA 0.2	2,4-D 0.2 C 2.0, K 1.0
Seedling tip	2ℓ	2,4-D 0.2, C 5.0	2,4-D 0.2 CM 10%
Seedling tip	1	IBA 0.5	CM 10%
Seedling tip	1ℓ	IAA 1.0, IBA 1.0, NAA 1.0, C 1.0, 2,4-D 1.0	Auxins 4.0[c] Cytokinins 5.0
Seedling tip	2ℓ	2,4-D 0.2, C 2.0, K 1.0	2,4-D 0.2, C 2.0, K 1.0
Seedling tip	1ℓ	2,4-D 2.5 CM 10%	2,4-D 0.2 CM 10%
Sucker tip	3ℓ	Adn 20.0	d
Sucker tip	3ℓ	None	d

[a] 1 Murashige and Skoog (1962), 2 Schenk and Hildebrandt (1972), 3 Knudson (1946) ℓ - liquid

[b] Abbreviations appear on page xiv. Undefined numbers are mg/ℓ.
Previous supplements were those used in the medium from which the culture was transferred to the medium in which root organogenesis occurred.

[c] Auxins were IAA, NAA, C, 2,4-D at 1.0 mg/ℓ each;
Cytokinins were B, D, K, Ben, Z at 1.0 mg/ℓ each, also 2 mg/ℓ GA

[d] Previous medium was 1 with D 15.0 mg/ℓ.

which caused embryogenesis of seedling stem tissue suspensions, consisted of maintaining the suspensions for over 3 months in Murashige and Skoog's basal medium supplemented with 1.25 mg/ℓ 2,4-D and 1 percent coconut milk. This balance of growth regulators caused the formation of the structures seen in Figure 9 of Chapter 1.

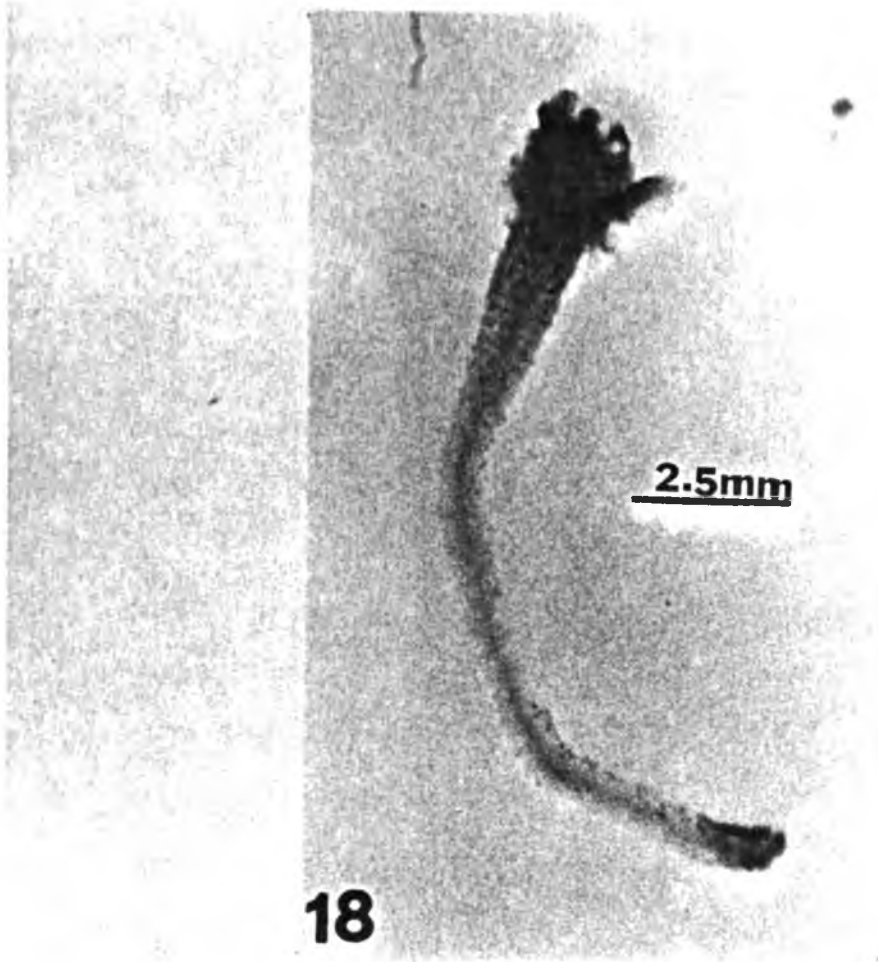
Another system that produced embryoids in several cell suspensions of seedling tip tissue consisted in maintaining the suspensions for 1 or 2 months in liquid Murashige and Skoog's or Schenk and Hildebrandt's basal media supplemented with 10 percent coconut milk. During this treatment meristemoids probably formed. The suspensions were then transferred to Schenk and Hildebrandt's basal medium containing 0.2 mg/ℓ 2,4-D, 2 mg/ℓ p-chlorophenoxyacetic acid, and 0.1 mg/ℓ kinetin. Over a period of 2 to 3 months, this low level of auxins and cytokinins caused the formation of structures with both a shoot and a root. (Figs. 18 and 19).

The third system to produce embryoids consisted in growing a cell suspension of seedling tip tissue in liquid Murashige and Skoog basal medium supplemented with 0.2 mg/ℓ 2,4-D and 10 percent coconut milk for a period of 1 month and then transferring it to the same basal medium with 0.2 mg/ℓ 2,4-D, but with 5 mg/ℓ p-chlorophenoxyacetic acid and 5 mg/ℓ kinetin. The high level of kinetin seemed to balance the auxins and permit the formation of both a root and a shoot.

The embryoids formed had no chlorophyll. In some, particularly in the early stages (Fig. 16, Chapter 2), the radical end contained abundant anthocyanin as judged by its color, but as morphogenesis proceeded, the primordial leaflets of the shoot and the root remained

Figure 18. Embryoid formed from seedling tip callus suspension in modified Schenk and Hildebrandt's basal medium.

Figure 19. Fused embryoids formed from seedling tip callus suspension in modified Schenk and Hildebrandt's basal medium.



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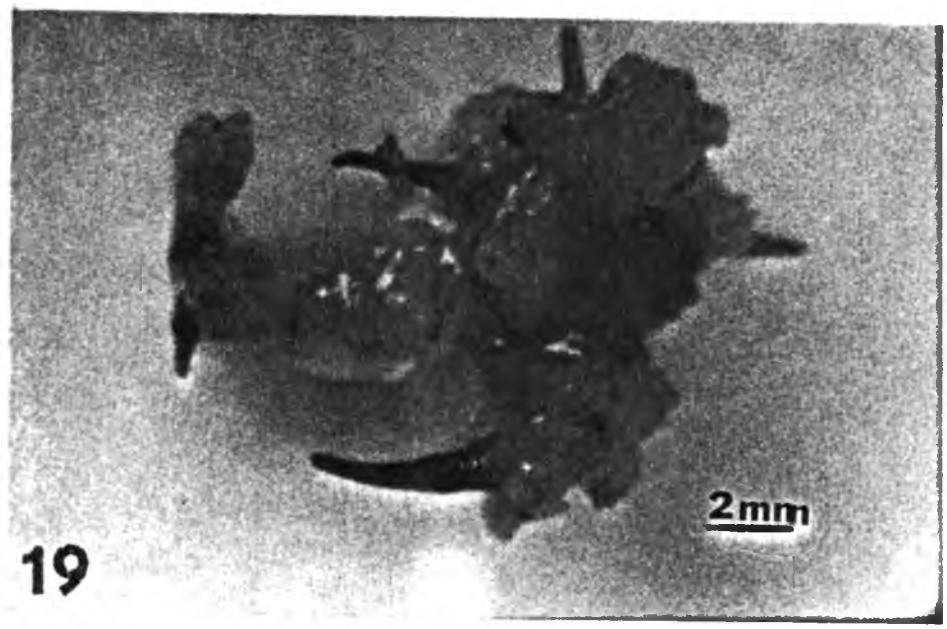

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colorless.

Numerous attempts were made to cause the embryoids to continue to develop, but all were unsuccessful. It was found possible to cause the roots to elongate by placing the embryoids in unsupplemented Murashige and Skoog basal medium. Also, the shoot portions were made to revert to callus and to form chlorophyll in the callus in media containing two auxins, two cytokinins, and coconut milk, as well as others containing four auxins and five cytokinins. Embryoids often formed in groups within a cell cluster and would separate from each other after formation (Fig. 19).

Embryoids were formed in erlenmeyer flasks, in t-tubes, and in nipple flasks. The apparatus did not seem to affect the degree of embryogenesis. The level of light did not have an effect on growth and development of the embryoids either. Several light levels were tried during the tests of various media. Embryogenesis occurred at levels of Cool-white light ranging from 16 to 42 $\mu E\ m^{-2}sec^{-1}$.

Organogenesis and elongation of shoots

Shoots were formed both on the callus surface and in the interior of callus and cell clusters on solid and in liquid media. Apical domes would form at the callus surface on agar (Fig. 20), or within cell clusters in suspension (Fig. 21). The domes at the callus surface on agar would elongate into the air, those formed in the callus interior would grow out through the callus until they emerged at the surface (Fig. 22). They would then elongate and leaf out (Fig. 23).

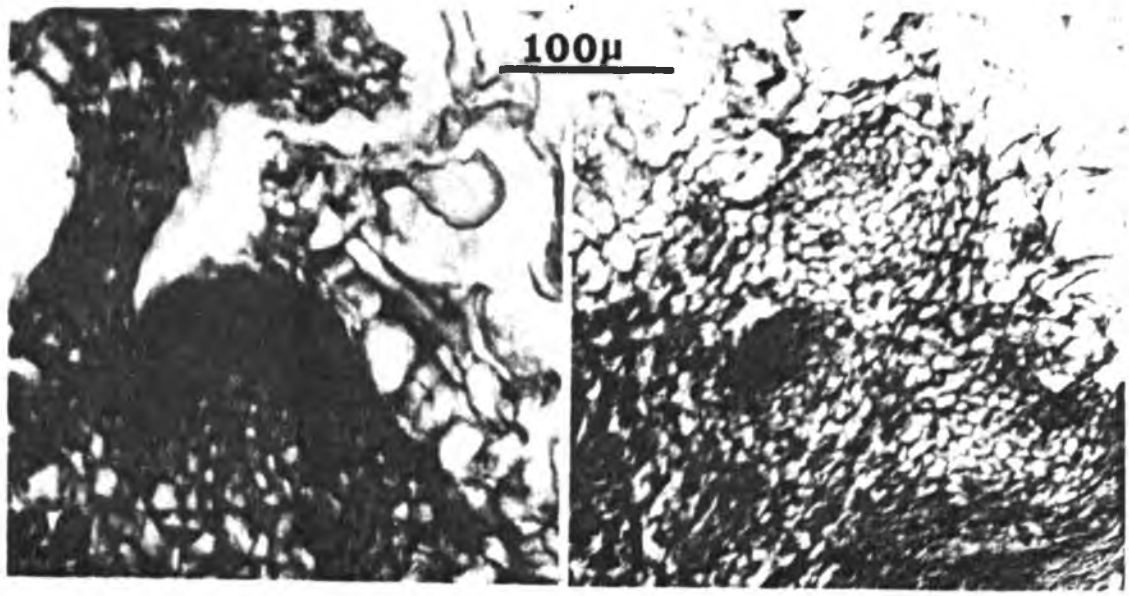
Organogenesis of shoots occurred frequently in slants at 40 to 50

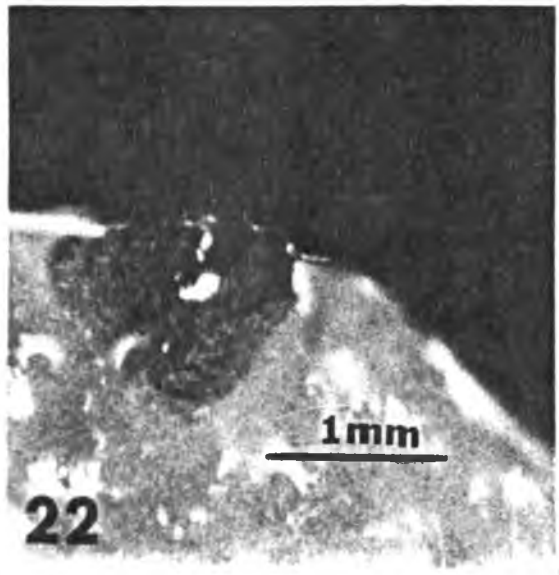
Figure 20. Apical dome formed on root sucker tip callus on an agar slant.

Figure 21. Apical domes formed internally in root sucker tip callus in liquid suspension.

Figure 22. Shoot tip that emerged from root sucker tip callus in liquid suspension just after transfer to a solid medium.

Figure 23. Leafy shoot formed from root sucker tip callus on agar slant.


100μ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22


23

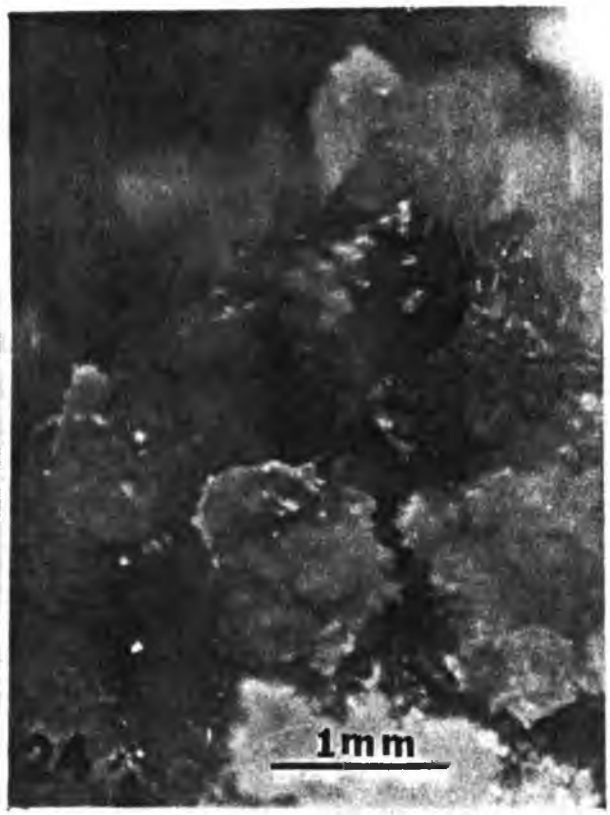
$\mu E\ m^{-2}sec^{-1}$ of Cool-white light. Elongation and leafing out of shoots required 56 to 88 $\mu E\ m^{-2}sec^{-1}$ of light supplied by combined Cool-white and Gro-lux lamps. Organogenesis of shoots in liquid media was most frequent in erlenmeyer flasks under 24 $\mu E\ m^{-2}sec^{-1}$ of Cool-white light. It also occurred in nipple flasks at 42 $\mu E\ m^{-2}sec^{-1}$ of Cool-white light.

Domes formed at the callus surface (Fig. 24) elongated more or less as normal shoots (Fig. 25). Leaflets would open and enlarge in a normal fashion. Those formed at the cell cluster surface in liquid media would often fail to elongate (Fig. 26), or do so in a very irregular manner (Fig. 27). However, some irregular morphogenesis also occurred in callus cultures on agar (Fig. 28).

Since shoot formation in callus and cell clusters usually takes place in peripheral tissues, the formation of apical domes endogenously, as illustrated in Figure 21 may be unique in plant tissue culture (M. Mapes, personal communication). They had an interesting developmental morphology. The development began with a rapidly dividing group of small non- or only slightly vacuolate cells, the meristemoid, (Fig. 29a) which began organization into a shoot meristem (Fig. 29b), and then formed a shoot meristem (Fig. 29c). As this elongated, the cells behind the meristem organized to form a procambium (Fig. 29d). Later, a definite cambial ring formed and radial division began (Fig. 29e). This continued as the pro-vascular system became recognizable (Fig. 29f). A complete ring formed and secondary thickening became obvious in the central vascular system (Fig. 29g). Still later, pit formation

Figure 24. Apical domes which were formed from seedling tip callus on agar slant of Murashige and Skoog's basal medium with 5 mg/ℓ benzyladenine.

Figure 25. Shoot elongation on Murashige and Skoog's basal medium with 1 mg/ℓ benzyladenine.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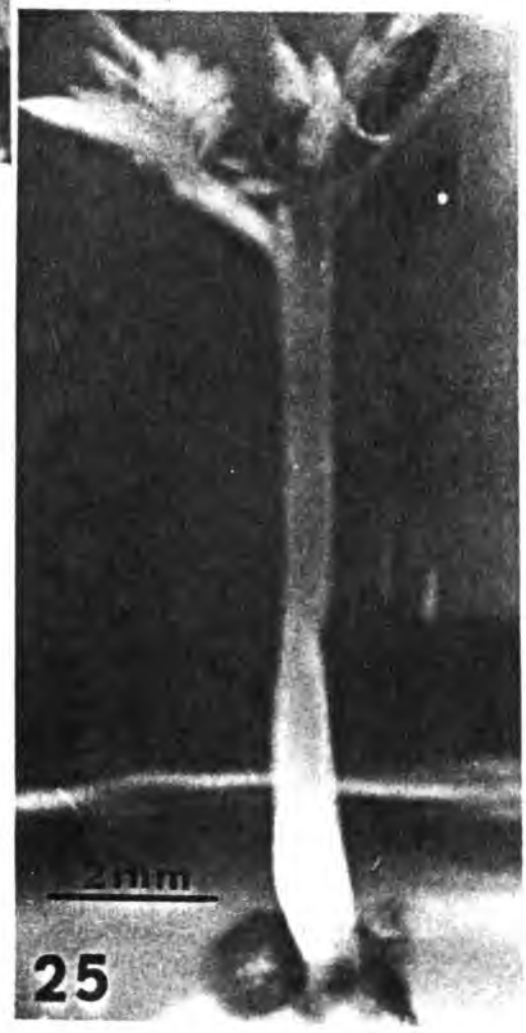
2mm
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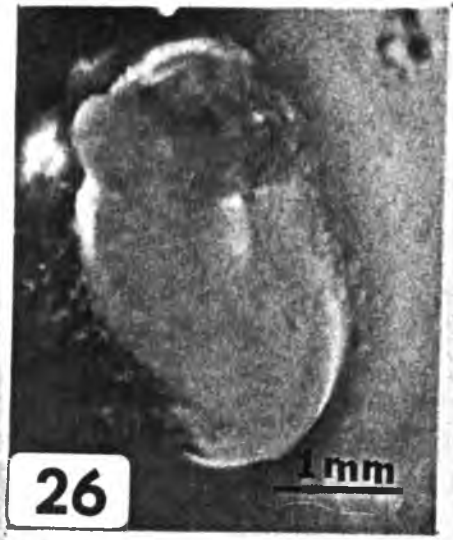
Figure 26. Shoot of root sucker tip callus formed in liquid culture. Note leaf primordia.

Figure 27. Shoot of root sucker tip callus formed in nipple flask suspension.

Figure 28. Shoot formation and beginning of elongation from seedling hypocotyl callus.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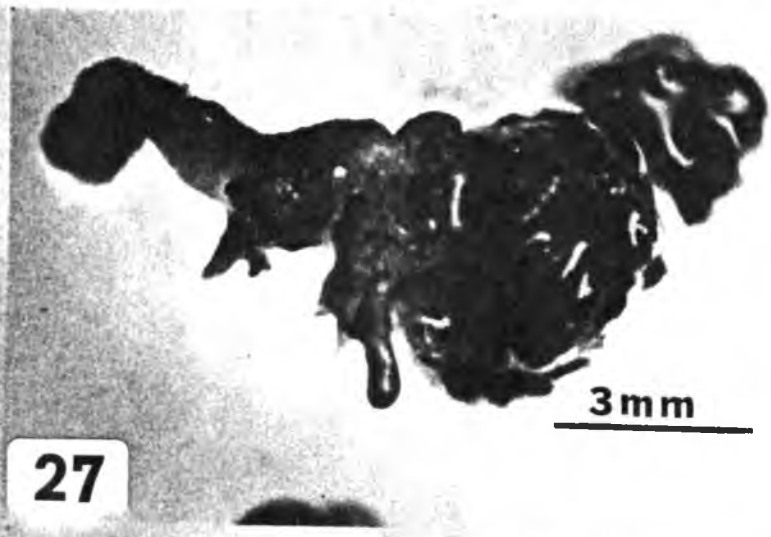
26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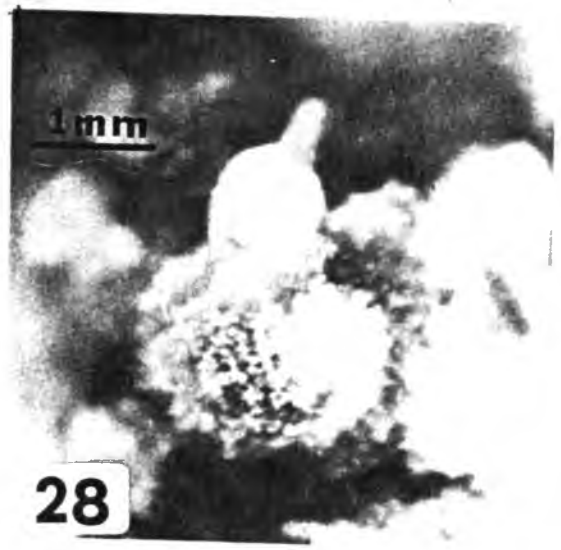
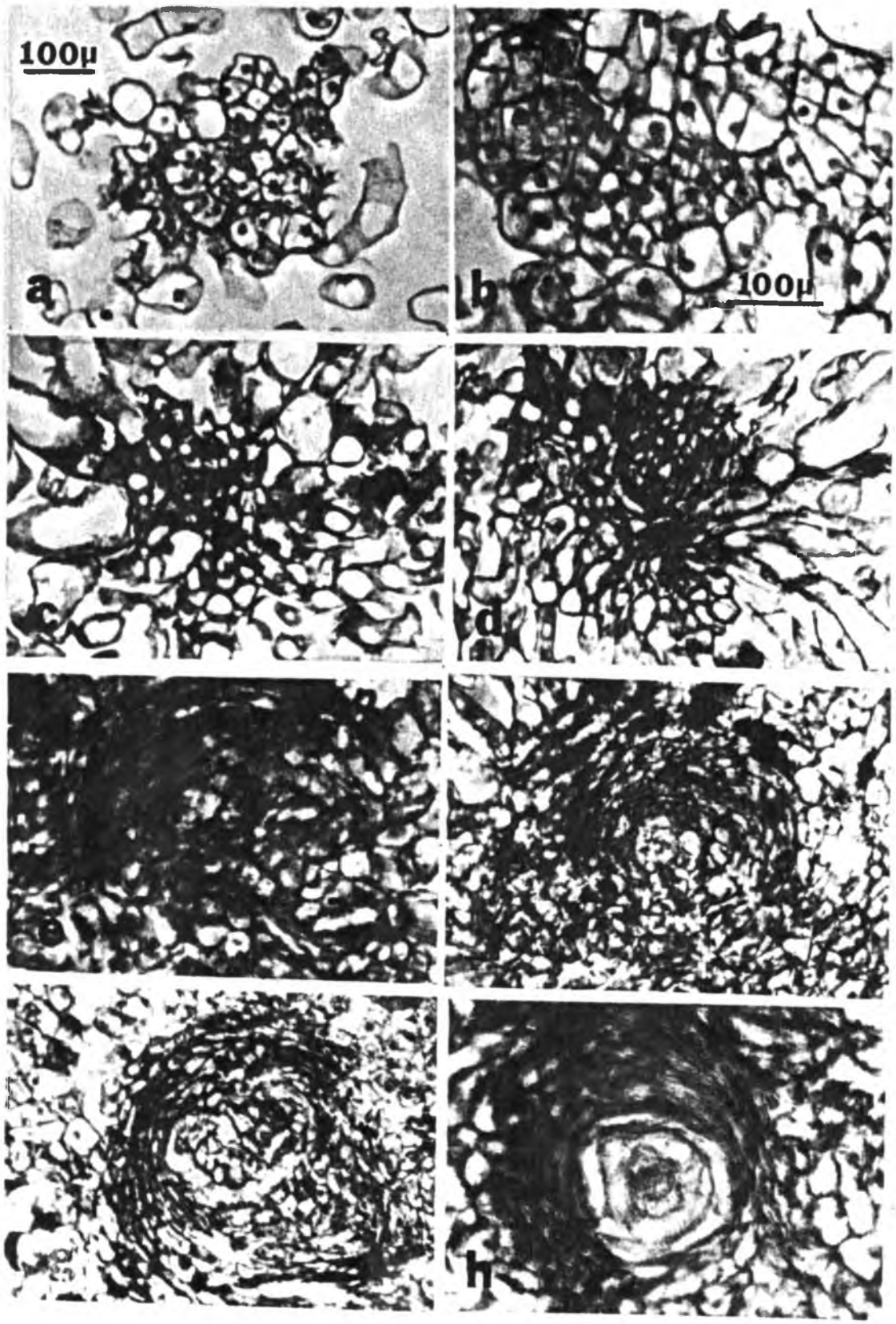

1 mm
28

Figure 29. A shoot begins to form within callus in liquid culture when a meristematic cluster (a) forms. Organization (b) follows rapidly, forming a shoot tip (c), which elongates leaving behind differentiating tissue beginning to form a stele (d). A cambial ring then begins to form (e), eventually producing a complete ring surrounding the provascular system which begins secondary thickening (f). This continues with advanced secondary thickening (g), until the stem with several phloem and xylem layers and a central core of vascular cells (h) with pits beginning to form is found deeply embedded in callus parenchyma.


100µ
a
b
100µ
d
h

commenced in the central vascular system and several layers of xylem and phloem formed from the cambial initials (Fig. 29h). All development occurred completely within the callus, with the shoot growing out through the callus parenchyma.

The media supplements that caused most shoot organogenesis to take place were benzyladenine, dimethylallyladenine, various combinations of these two, and the complex mixtures of four auxins, five cytokinins, and gibberellic acid referred to previously.

Tissue from six different seedling tips, a seedling hypocotyl, a root sphaeroblast meristem, and four root sucker tips have regenerated shoots. However, almost all the shoots produced formed from only two cultures, one of seedling tip callus, and the other of a root sucker tip from a superior tree. Different growth regulators were required to cause organogenesis of different tissue sources.

A list of tissues and the media for which organogenesis occurred showed several inconsistencies (Table 13). One clone of seedling tip callus organized into shoots primarily in the presence of benzyladenine, whereas a clone of root sucker tip callus and cell suspensions only did so in the presence of dimethylallyladenine. Other clones of seedling tip callus (Numbers 7, 9, Table 13) formed shoots in media containing coconut milk rather than these cytokinins, and one other clone of root sucker tip tissue (Number 35) formed a shoot in response to kinetin. Most shoots formed on or in media containing little or no auxin. However, two suspensions (Numbers 10, 35) differentiated into shoots in media intended for callus growth

TABLE 13

TISSUE SOURCES AND MEDIA THAT RESULTED IN SHOOT REGENERATION

Reference number	Tissue	Medium[a]	Growth regulators[b]
1	Seedling hypocotyl callus	1	B 5.0
2	Seedling tip callus (1)	1	B 5.0
3	Seedling tip callus (1)	1	B 10.0
4	Seedling tip callus (1)	1	B 10.0, K 5.0
5	Seedling tip callus (1)	1	2.0 ea B, Ben, K, D, Z
6	Seedling tip callus (1)	1	1.0 ea B, Ben, K, D, Z + CM 10%
7	Seedling tip callus (2)	1	2,4-D 2.5, Adn 20.0, CM 10%
8	Seedling tip callus (3)	1	B 5.0
9	Seedling tip callus (4)	1[c]	2,4-D 0.2, Adn 20.0, CM 10%
10	Seedling tip suspension (5)	2	2,4-D 0.2, C 2.0, K 1.0
11	Sphaeroblast meristem suspension	1	0.2 ea IAA, IBA, NAA, C, 2,4-D 1.0 ea B, BT, D, K, Z + GA 2.0
12	Root sucker tip suspension (1)	1	1.0 ea IAA, C, NAA, 2,4-D B, Ben, D, K, Z + GA 2.0
13	Root sucker tip callus (1)	1	D 15.0
14	Root sucker tip suspension (1)	1	D 15.0
15	Root sucker tip callus (1)	1	2,4-D 0.2, D 15.0
16	Root sucker tip suspension (1)	1	2,4-D 0.2, D 15.0
17	Root sucker tip callus (1)	1	B 5.0, D 10.0
18	Root sucker tip suspension (1)	1	B 5.0, D 10.0
19	Root sucker tip suspension (1)	1	IAA 2.0, NAA 2.0, B 2.5, D 2.5
20	Root sucker tip callus (1)	1	2.0 ea B, Ben, K, D, Z
21	Root sucker tip suspension (1)	1	2,4-D 0.2, B 0.5
22	Root sucker tip suspension (1)	1	D 1.0, Adn 20.0
23	Root sucker tip callus (1)	1	D 15.0, Adn 20.0
24	Root sucker tip callus (1)	1	B 10.0, D 10.0
25	Root sucker tip suspension (1)	1	B 10.0, D 10.0

TABLE 13. (Continued) TISSUE SOURCES AND MEDIA THAT RESULTED IN SHOOT REGENERATION

Reference number	Tissue	Medium[a]	Growth regulators[b]
26	Root sucker tip suspension (1)	1	B 1.0, D 1.0
27	Root sucker tip callus (1)	1	B 10.0, D 20.0
28	Root sucker tip suspension (1)	1	IAA 2.0, NAA 2.0, B 4.0, D 6.0
29	Root sucker tip suspension (1)	1	B 2.0, D 3.0
30	Root sucker tip suspension (1)	1	B 4.0, D 6.0
31	Root sucker tip suspension (1)	1	D 10.0
32	Root sucker tip suspension (1)	1	2,4-D 0.2, D 10.0
33	Root sucker tip suspension (1)	1	NAA 2.0, D 15.0
34	Root sucker tip suspension (1)	1	IAA 2.0, NAA 2.0, B 2.0, D 3.0
35	Root sucker tip suspension (2)	2	2,4-D 0.2, C 2.0, K 1.0
36	Root sucker tip callus (3)	1	1.0 ea IAA, NAA, C, 2,4-D, B D, Ben, K, Z + GA 2.0
37	Root sucker tip callus (4)	1	B 0.1

[a]1 - Murashige and Skoog (1962)
2 - Schenk and Hildebrandt (1972)

[b]Abbreviations on page xiv, numbers are mg/ℓ

[c]NH_4 NO_3 absent from basal medium

which were quite high in auxin.

The media on which organogenesis occurred (Table 13) were in many instances the last of an intentional sequence of media on or in which the tissue was placed. The prior treatments may have played a role in organogenesis. For example, the calluses of numbers 1 and 2 were each grown first on media containing 2,4-D and coconut milk and then on media supplemented with coconut milk alone before being placed on the benzyladenine media on which organization occurred. It is believed that meristemoids were formed in the coconut milk treatments and were caused to differentiate into shoot meristems by the benzyladenine. H. E. Street (M. Mapes personal communication) has suggested that the 2,4-D treatment may be required to induce the RNA synthesis that resulted in triggering meristemoid formation.

The callus of numbers 3 and 4 was grown on the complex of four auxins, five cytokinins, and gibberellic acid which has been referred to repeatedly. This medium seemed to induce development of meristemoids, or the potential for later shoot formation, because transfer of the callus to the high cytokinin media of both 3 and 4 resulted in rapid organization into multiple shoot clusters. Interestingly, part of this same callus was grown for a month on a medium containing benzthiazolyloxyacetic acid, rather than benzylaminobenzimidazole (Ben), before being placed on the same shoot organizing media as the others. This small change of cytokinins caused necrosis and death to occur in the tissue after transfer, rather than shoot formation as occurred with the callus transferred from the medium containing Ben.

Murashige (1974b) has noted that shoot formation is generally inhibited by 2,4-D. This was not found to be so with root sucker tip tissue. Essentially as many shoots formed in both agar and liquid cultures with 2,4-D present as without. In one instance (Number 7), a shoot developed on a medium with the rather high level of 2.5 mg/ℓ 2,4-D.

Perhaps the most unusual result was that shoots formed in cell suspension cultures at the same levels of growth regulators as were used for agar callus cultures. It would be expected that tissue in intimate contact with a liquid medium would be much more sensitive to growth regulator levels than callus on the surface of gelled media. The cells that differentiated into shoots in the callus were separated from the agar media by hundreds of cells, whereas those in the liquid media were in almost direct contact.

Despite this, callus cultures such as numbers 13 and 15 (Table 13) formed shoots similar to and at about the same frequency as suspensions such as 14 and 16. Media with low levels of cytokinins such as number 26 also caused shoots to form. It can only be inferred that this particular culture was very insensitive to the level of cytokinin.

The first shoot to form was number 1, from seedling hypocotyl tissue. One month after being formed, it was placed on a medium containing 10 percent coconut milk on which it elongated and formed leaflets. It also formed a second basal shoot which was separated from the first. Both these shoots eventually died.

The second shoot that formed was number 2, from seedling tip

callus. This was also transferred 1 month after formation to basal medium with 10 percent coconut milk on which it formed a shoot cluster (Fig. 30). After another month, it was placed on basal medium with 1 mg/ℓ benzyladenine, which caused the shoot to elongate rapidly and form leaflets (Fig. 31). It was grown on this medium for several months and then transferred to basal medium without growth regulators.

Another piece of the same seedling tip callus, which had been rapidly increased on the medium containing four auxins, five cytokinins, and gibberellic acid, proliferated into multiple shoots when placed on a slant of basal medium containing 10 mg/ℓ benzyladenine (Number 3) and also another slant with 10 mg/ℓ benzyladenine and 5 mg/ℓ kinetin (Number 4). The shoots formed on the 10 mg/ℓ benzyladenine medium were transferred to a 1 mg/ℓ benzyladenine medium, then to a 10 percent coconut milk medium, then back to 1 mg/ℓ benzyladenine and so forth through three cycles, each transfer lasting for 1 month. This caused multiple shoots to proliferate, elongate, and form leaflets, after which the culture was cut apart and subcultured to other 1 mg/ℓ benzyladenine plates and slants. This culture has produced more than 100 shoots and continues to do so.

The shoots formed on the benzyladenine-kinetin medium were transferred first to a 10 percent coconut milk medium, then to 1 mg/ℓ benzyladenine and back to coconut milk in just the opposite sequence to the other shoots. All eventually died, either because of the kinetin used in shoot induction or because of the reverse sequencing of coconut milk and benzyladenine.

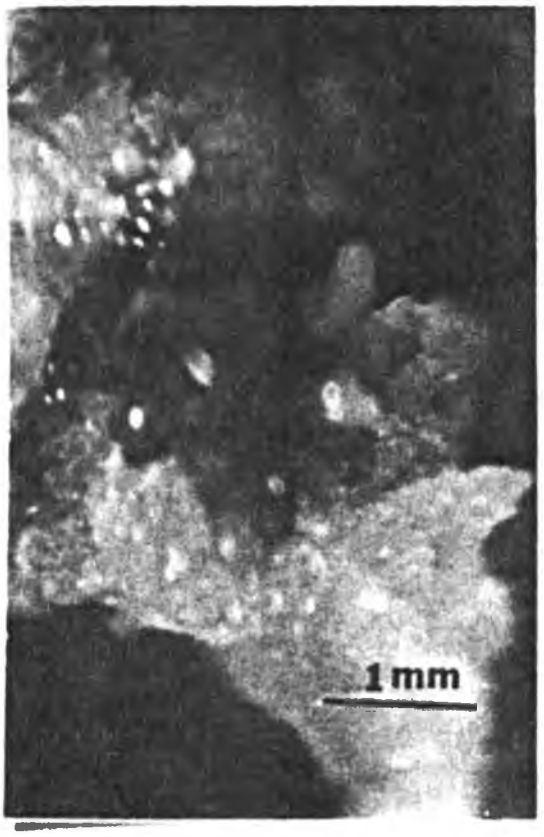
Another shoot was formed from a different seedling tip callus

Figure 30. Emerging domes and leaflets of seedling tip callus forming a meristematic cluster.

Figure 31. Differentiating shoot of seedling tip callus on basal medium with 1 mg/ℓ benzyladenine.

Figure 32. Shoot of root sucker tip callus beginning to form leaflets.

Figure 33. The first shoot of root sucker tip callus to leaf out and enlarge.



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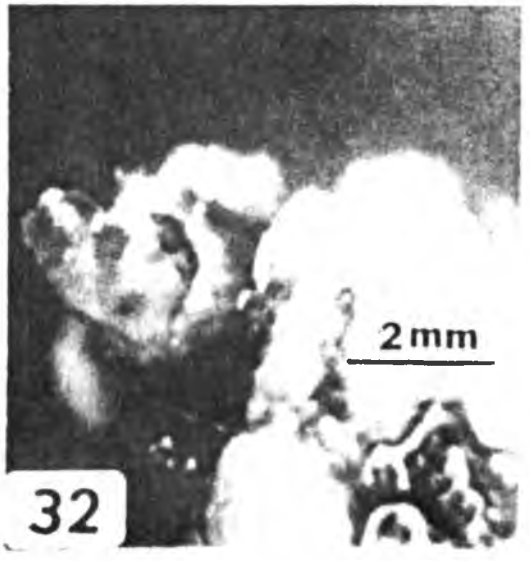

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33

(Number 8) by using the same procedures used to obtain shoot induction in number 2. This indicated that the process was repeatable. Other shoots have formed from seedling tip callus and suspensions given different processing (Numbers 5, 6, 7, 9, and 10).

The first shoot to form from forest tree tissue in a suspension culture did so in culture number 12. Several shoots similar to that in Figure 26 formed in this medium, but did not develop further despite transfer to a number of different media.

The suspension culture was subcultured for several months in both solid and liquid media and eventually began to differentiate into shoots again on and in media 13 and 14 and in medium 19. The same tissue in later subcultures produced shoots on and in many other media (Table 13). Some of these shoots elongated and formed leaflets in liquid media, but most only elongated on agar media or when transferred from liquid to agar media. Elongation of the hundreds of shoots that have formed has occurred most frequently on basal medium without growth regulators, but also occurred frequently on one-half strength basal media with 10 rather than 30 g/ℓ of sucrose. No consistency has yet been achieved in obtaining shoot elongation of this tissue source despite hundreds of trials of media and environmental conditions.

The shoots that emerged from root sucker tip callus looked much like those of seedling tip callus (Fig. 32). When they elongated, however, they did not develop the erect form of the seedling tip callus shoots (Fig. 33). Shoots from cell suspension cultures of root sucker tip tissue elongated and formed leaflets similar to those

formed on callus (Fig. 34).

Other shoots have formed from root sucker tip callus and suspensions, but not when subjected to the same treatments that caused massive proliferation of the one root sucker tip culture.

Rooting of Shoots

The first shoot to root was number 2 (Table 13). The root that formed (Fig. 35) was found emerging from the basal callus of the shoot only 3 weeks after transfer of the shoot from basal medium to a medium with 0.2 mg/ℓ indolebutyric acid. The root grew rapidly when the plantlet was transferred back to basal medium and other shoots began to grow from the callus at the base of the original shoot (Fig. 36). Eventually, this plantlet was cut apart into seven separate shoots, but in so doing the root was severed, and all the shoots eventually died *in vitro*.

The next rooting occurred on a shoot formed in number 3, but only after many trials. Many shoots from the proliferating callus formed in medium number 3 were grown on Murashige and Skoog's basal medium supplemented with 1 mg/ℓ benzyladenine. These shoots elongated quite well, but during a period of 10 months, none rooted when transferred to media containing 0.2 mg/ℓ indolebutyric acid, the growth regulator which had previously caused rooting. Media containing indoleacetic acid and napthaleneacetic acid at various levels did not cause root formation either. Finally, a shoot that had been grown on basal medium without growth regulators for a month quickly rooted after transfer to a 0.2 mg/ℓ indolebutyric acid medium. It was noted that the original rooting had occurred after a similar sequence of

Figure 34. Shoot formed in suspension culture elongating on agar slant.

Figure 35. Root emergence from underside of seedling tip callus shoot.

Figure 36. Rooted plantlet of seedling tip callus.


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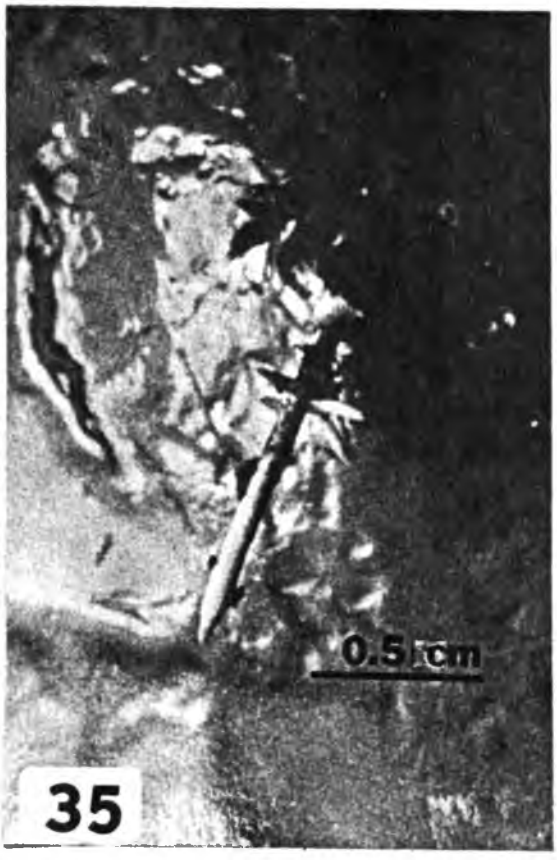
1cm


0.5cm
35


36
1cm

transfers, and this proved to be the key to rooting.

The benzyladenine inhibited rooting and had to be removed or made inactive by holding the shoot on unsupplemented basal medium for at least 3 weeks. Once this was learned, over 50 percent rooting of shoots *in vitro* began to be achieved. Several trials using a number of levels of indolebutyric acid and napthaleneacetic acid indicated that the most frequent rooting occurred on media containing 0.3 mg/ℓ of indolebutyric acid.

A large variation in elongation was apparent among the shoots of the seedling tip clone even when grown in the same flask (Fig. 37). The size differences did not greatly influence rooting capacity although there was a tendency for the tallest seedlings to root less frequently than those of medium to small size.

The roots formed were also variable. Occasionally, they would grow from the stem above the basal callus (Fig. 38), but much more frequently they grew out through the basal callus on its underside. Some roots grew rapidly to very large size, showing the variation inherent within the clone (Fig. 39).

Root growth was increased by exposure of the shoot to combined Cool-white and Gro-lux light in excess of 80 $\mu E\ m^{-2}sec^{-1}$ although it also occurred regularly at Cool-white levels as low as 50 $\mu E\ m^{-2}sec^{-1}$. Activated charcoal was tried to make root elongating media opaque in one experiment (Fig. 40). This was found to improve the root growth of plantlets with long roots, but prevented growth of small poorly developed roots. The medium used for elongation was that of Murashige and Skoog without supplements. The gain with the use of activated

Figure 37. A flask full of shoots of various sizes growing in unsupplemented basal medium; 250 mℓ flask.

Figure 38. "Aerial" roots growing from a plantlet on basal medium with 0.2 mg/ℓ indolebutyric acid.

Figure 39. A very large root system growing from a plantlet on basal medium with 0.2 mg/ℓ indolebutyric acid, in 250 mℓ flask.

Figure 40. A trial of darkening medium with activated charcoal to improve root growth in 250 mℓ flask.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charcoal was judged insufficient to justify its use.

Another rooting medium that was successful with shoots of seedling tip callus was one containing 0.5 mg/ℓ napthaleneacetic acid. However, rooting was much less frequent on this medium than on media containing 0.2 or 0.3 mg/ℓ indolebutyric acid.

Most of the shoots were placed on the rooting medium with a lump of callus at their bases, but many were also excised completely from any callus. Only those shoots on a basal callus formed shoots. The basal callus was evidently necessary to "filter" or "modulate" the supply of nutrients and growth regulators to the shoot in forms suitable for root induction. The roots usually grew from that portion of the stem imbedded in the callus.

The seedling tip callus culture has continued to proliferate and produce shoots on the 1 mg/ℓ benzyladenine medium for over 2 years. Rooting has become easier with the passage of time. For the first 6 to 8 months, no rooting occurred except after the shoots had been transferred to the indolebutyric acid medium. Then roots began to form on occasional shoots while still in the unsupplemented basal medium prior to transfer to the rooting medium. Most recently almost one-third of the roots have formed on the basal medium treatment, and in one flask, roots were produced from the stems of two shoots in a 1 mg/ℓ benzyladenine medium, the medium that had formerly inhibited rooting.

Roots have also formed from root sucker tip shoots, both on agar and in liquid suspension culture. These roots formed primarily in, or on Knudson's (1946) basal medium either with or without 20 mg/ℓ adenine.

They also formed in one liquid culture of Murashige and Skoog's basal medium supplemented with 5 mg/ℓ benzyladenine and 10 mg/ℓ dimethylallyladenine, and on several slants of unsupplemented basal medium. So far, the roots have not elongated beyond about 0.5 cm in length on any of numerous media tried for this purpose.

Aftercare of Plantlets

The first five rooted plantlets removed from the flasks were potted in sterile potting mix (1 part each by volume of vermiculite, peat moss, and perlite) in plastic tubes and placed under intermittent mist (Fig. 41). They did not grow during a 2-month period and after removal from the mist chamber, wilted and died. It was found on examination that the roots had not grown.

A second group of six rooted plantlets, two of which had been grown in flasks of unsupplemented medium with activated charcoal (Fig. 40), two from a flask of sterile potting medium, and two from flasks of unsupplemented medium without sucrose were given the same treatment. Again, all desiccated and died after removal from the mist.

Another group of ten plantlets were placed in flasks of full-strength liquid Hoagland's solution (1950), five of which were covered with aluminum foil (Fig. 42) and five of which were left exposed to the light (Fig. 43). Four of the five plants in each treatment became desiccated and died during the first week of exposure, but the two that were left developed large root systems during a period of 1 month. The roots grown in the dark were much more branched than those grown in the light (Fig. 44).

Figure 41. Five seedling tip callus 1 plants in tubes of potting mix under mist.

Figure 42. Rooted plant in flask of Hoagland's solution.

Figure 43. A test of root growth in Hoagland's solution in covered and uncovered flasks.

Figure 44. Root systems developed in Hoagland's solution. Light on left, dark on right.


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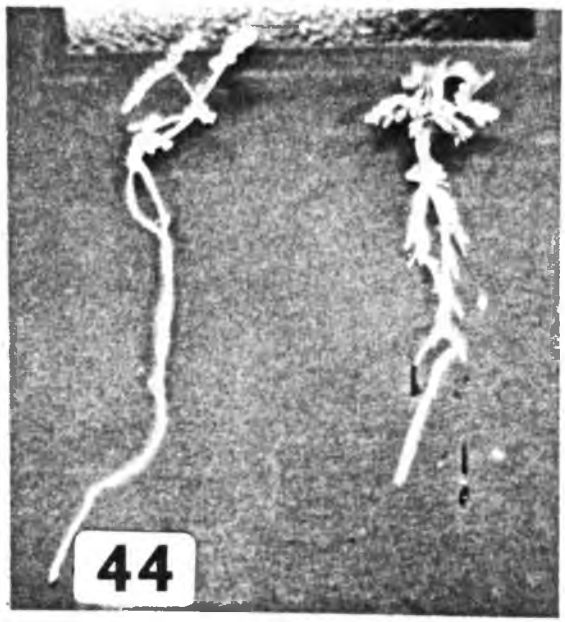
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These plants were then potted, placed under mist for 1 month and then removed. Both survived and grew into normal trees.

After much trial and error it was determined that desiccation of plantlets removed from sterile culture and placed in flasks of Hoagland's solution could be prevented by encasing each flask in a clear polyethylene bag for a 1-month period of root growth. Then after potting, the plants were found to almost always survive if they were again kept in polyethylene bags for another month before exposure. Lastly, the potted plants were found to grow more rapidly if exposed to 120 μE $m^{-2}sec^{-1}$ of Gro-lux light than under 90 μE $m^{-2}sec^{-1}$ of incandescent light.

Microscopic examination of the roots grown in agar media and those grown in Hoagland's solution showed that the roots from the liquid had developed root hairs. These were not present in the roots from agar.

The plants were grown in small plastic tubes until they developed white succulent buds and at least three leaves. They were then transplanted to 1-quart plastic bags of potting mix. They were fertilized regularly with a 20-20-10 liquid fertilizer and grown indoors under bright light until about 10 to 15 cm tall after which they were transferred to a greenhouse to harden in the sunlight for 2 months before planting in the field.

Clonal Variation

Within the clone of koa shoots, plantlets, and semi-autotrophic plants, there was a great deal of variation. Not only did shoots grow from different locations with respect to the callus surface, but they

also reacted differently to the same stimuli. Some elongated more than others (Fig. 37) in the same environment. Some rooted, others did not. Some formed large roots, others small roots. Some produced small, chlorotic plants with pointed, narrow leaflets, while others produced plants essentially identical to natural seedlings (Fig. 45).

Variation in root growth and development was the most notable type of variation. Although there was some reaction to medium--thin roots formed on unsupplemented basal media, thick roots on media with indolebutyric acid--even on the same medium roots of some ramets (plants of a clone) would remain stubs, while others elongated well.

It was noted that as roots developed, the shoot took on normal characteristics, indicating that the variation in stem length and leaflet shape was associated with condition of the root. After a period of growth in the greenhouse, the plants that had been quite variable in sterile culture and the early stages of aftercare took on a uniform, normal appearance (Fig. 46).

When planted out in the field, the tissue culture-produced trees looked just like young natural seedlings (Fig. 47). It is therefore uncertain at this time whether significant clonal variation has resulted from the tissue culture propagation process.

Chromosome counts were made in only two of five root tip squashes attempted. The root tips, taken from Hoagland's solution cultures, were stained with Carnoy's solution (Klein and Klein 1970), hydrolyzed, and squashed using standard techniques. The two counts gave a diploid number ($2n = 26$). Koa is said to be a tetraploid (Whitesell 1964).

Four tissue culture-produced trees have formed phyllodes (Fig. 48).

Figure 45. Leaflets of the ramets of this clone were quite variable in size and form when first formed.

Figure 46. Young trees in the greenhouse developed into uniform size and form after 3 to 5 months.

Figure 47. After outplanting, the trees looked quite normal.

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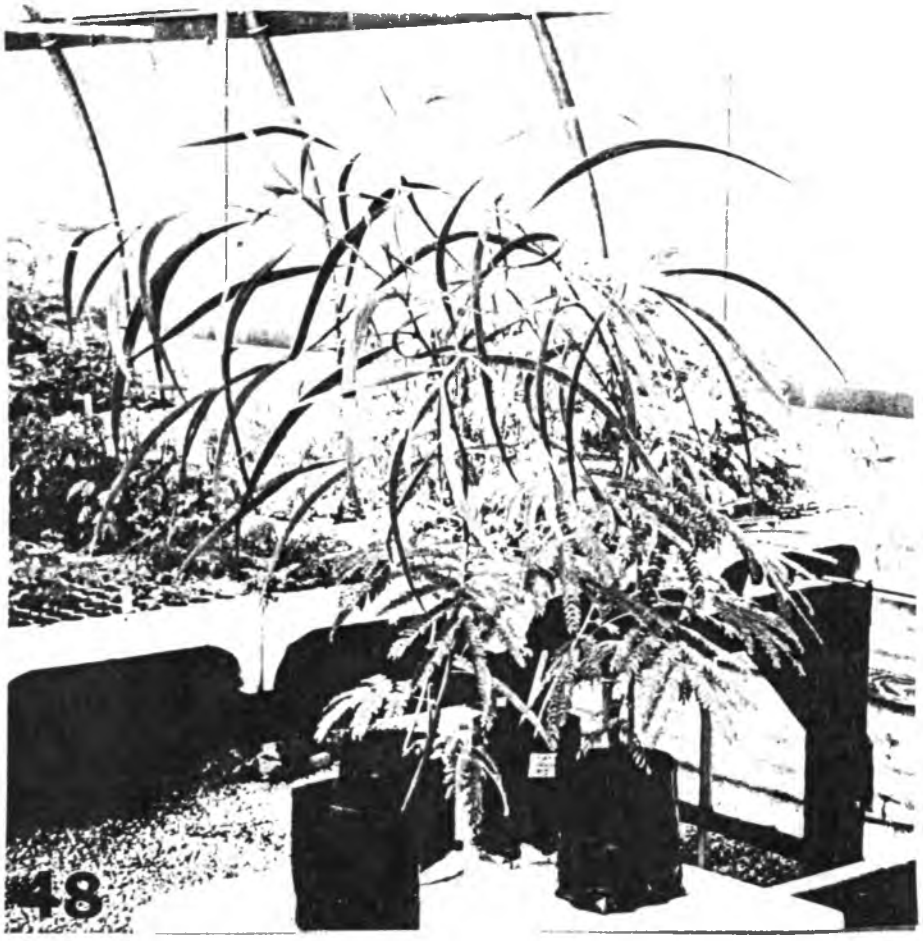
Figure 48. Tissue culture-propagated trees formed phyllodes in the warm greenhouse indicating the genetic message for maturing was present.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This indicated very strongly that the genetic message to reproduce a mature tree was preserved through the callusing and shoot regeneration process. It also indicated ontogenetic change was not arrested by the process. The phyllodes were "forced" by growing the plants in a very warm, humid environment. Phyllodes grown in this environment have been found to grow long and narrow as demonstrated in Figure 58.

Discussion

Torrey's (1966) proposed meristemoid system would fit nicely with the organogenesis observed in koa tissue. Roots were formed when meristemoids formed in coconut milk- and cytokinin-containing media were induced to become root meristems by placement in high auxin media. Embryoids were formed when the meristemoids were induced to form both root and shoot meristems, by being provided low, and nearly equal levels of auxin and cytokinin at the same time. Shoot meristems were induced by high levels of cytokinin with very small amounts, or in some instances, no auxin.

The multi-celled embryoids that formed in koa cell suspensions were similar to those produced from carrot tissue in Steward's laboratory (A. Krikorian, personal communication). They were much more advanced morphologically than the structures called embryoids by Winton and Verhagen (1977) and others (Durzan and Lopushanski 1975). In fact, they are believed to represent the first true embryoids to be formed from a forest tree in culture.

Meristemoids were formed in media containing coconut milk (Fig. 29a), and also in the media with four auxins and five cytokinins. This was demonstrated when the cultures from these media developed

shoots after transfer to high-cytokinin media. The mixed auxins and cytokinins seemed to supply requirements for meristemoid development similar to those supplied by the undefined organic complex, coconut milk.

Further evidence that the auxin-cytokinin complex that was developed had a similar action to coconut milk was provided by the fact that both caused chlorophyll formation in colorless cultures, and both caused cell clusters to form by rapid division in cell suspensions. The auxin-cytokinin mixture seemed to provide augmented coconut milk effects.

As was pointed out in the literature review, phenolics may have a serious inhibitory influence on rooting as well as other forms of organogenesis. It is believed that one of the major reasons koa tissue has been successfully regenerated is that it did not produce high levels of phenolics. There was no need to use citric acid (Murashige 1974b) or other oxidants in an attempt to avoid phenolic buildup in cultures of koa.

Perhaps partly as a result of its low phenolic output in culture, and partly because of the development of the auxin-cytokinin medium, koa was induced to produce shoot meristems, stems, and leaves in liquid culture. No other research was found in which these events had occurred with forest tree tissue. This cell suspension system has a large advantage over the callus system for mass propagation because it is only necessary to remove and plate out the shoots as they form. Time consuming callus division and subculturing is avoided.

The need for sequential treatments in tissue culture was amply

illustrated by the behavior of the seedling tip callus which had to be alternately subcultured to 1 mg/ℓ benzyladenine and 10 percent coconut milk several times in order to cause proliferation of shoots. Some such stepwise procedure may be needed to cause the root sucker tip shoots to form plantlets. Sequential treatments were similarly essential in leaching or changing the benzyladenine from the shoots in unsupplemented media so that they would root and also in causing agar-grown roots to become functional.

In addition to the formation of embryoids and shoots in liquid culture, another unique or nearly unique development of this research was the formation of shoots within callus and cell clusters rather than upon the surface. This was particularly unusual in that it occurred on and in the same media that caused shoot formation at the tissue surface. Thus it was not obviously the result of "amelioration," or modification of growth regulators from the medium by passage through membranes from cell to cell. The formation of shoots from cells in direct contact with the media suggests that some other mode of modification took place in shoot induction, or that the tissue was very tolerant of cytokinin levels.

Two clones of tissue were induced into the state of mass shoot proliferation desired for tissue culture propagation. A major problem is that they were caused to do so by two different systems. One clone reacted favorably only to benzyladenine. Dimethylallyladenine caused it to become necrotic. The other clone reacted as desired only when dimethylallyladenine was present, and no shoots formed with benzyladenine alone. So far, this clone has been the only one to have

produced shoots in response of dimethylallyadenine. However, several clones have produced at least one shoot in response to benzyladenine. If each clone requires a different system, tissue culture propagation of koa will, of course, be impractical.

The ages of the two cultures producing large number of shoots is also rather unusual. One, the seedling tip culture, is now nearly 4 years old. The root sucker tip culture is over 2 1/2 years old. Neither has shown any sign of ceasing to form shoots.

Still another unsolved problem, though in this case, a desirable one, is why rooting is now occurring in the unsupplemented media used to remove the benzyladenine. Since auxin is required to form roots, it must be assumed that auxins have begun to be produced in the buds and leaves of the shoots in culture. This suggests that a mass change in the shoots in many different flasks has been going on, perhaps in relation to the age of the culture.

The function of the Hoagland's solution treatment in causing non-functional roots to develop root hairs and become functional is not clear. It is probably not a matter of oxygenation because no agitation or oxygenating of the Hoagland's solution has been required. Murashige and Skoog's basal medium without sucrose was substituted for Hoagland's solution and did not result in normal root growth, so it is possible that the chemicals in the Hoagland's solution are the cause of improved root growth. Results with root induction in other cultures, such as the rooting of root sucker tip tissue on low sucrose and Knudson's media, suggest that nutrition may play an important role in root growth.

The change of appearance in the tissue culture-propagated plants as they were gradually converted to autotrophic growing conditions was not unique for the species or procedure. Similar behavior has been reported for tissue culture-propagated *Pseudotsuga menziesii* by Wochok and El-Nil (1977) and for *Pinus taeda* by Mott (R. Mott, personal communication). It suggests that the selection that occurs in subculturing, and the consequent variation among subcultures, may not be particularly serious. However, this must be determined by long-term progeny tests of tissue culture-propagated clones.

Summary and Conclusions

It is clear that the objective of this work, to vegetatively propagate mature forest-grown trees by tissue culture, has not yet been met. The only trees so far grown and field-planted have been of a laboratory germinated seedling of unknown genotype. However, even these results are quite notable in that they represent a degree of success that is still very rare in the field of woody-plant propagation by tissue culture.

Acacia koa can be propagated *en masse* by tissue culture using seedling tips as the source material--this has been demonstrated. It may also be possible to propagate mature koa trees that form root suckers, or adventitious shoots. It has been demonstrated that such tissue can be made to produce large numbers of shoots in sterile culture, but complete rooted plants have not yet been grown from tissue taken from mature trees.

The procedure that has been developed for tissue culture propagation of koa seedling tip tissue is as follows (using the

previously described laboratory conditions):

1. Grow callus or a cell suspension from sterile shoot tips cleaned of outer leaf primordia on agar slants of Murashige and Skoog's (M) basal medium containing 1 mg/ℓ each of indoleacetic acid, napthaleneacetic acid, p-chlorophenoxyacetic acid, 2,4-D, benzyladenine, benzylaminobenzimidazole, dimethylallyadenine, kinetin, and zeatin and 2 mg/ℓ gibberellic acid, or in liquid Schenk and Hildebrandt's basal medium with 0.2 mg/ℓ 2,4-D, 2.0 mg/ℓ p-chlorophenoxyacetic acid, and 0.1 mg/ℓ kinetin.
2. Place callus or cell aggregates on agar medium of the above mixed auxins and cytokinins to increase, or on solid M basal medium with 10 percent coconut milk.
3. Place callus on solid M basal medium with 5 or 10 mg/ℓ benzyladenine added.
4. Excise shoots that form, with some basal callus, and place on solid M basal medium with 1 mg/ℓ benzyladenine for 1 month.
5. Place shoots on solid M basal medium with 10 percent coconut milk for 1 month.
6. Repeat steps 4 and 5 until shoots proliferate.
7. Increase proliferation and elongation on solid M basal medium with 1 mg/ℓ benzyladenine.
8. Place elongated shoots individually, but with basal callus on solid M basal medium without additives for 1 month.
9. Place shoots on rooting medium, solid M basal medium with 0.3 mg/ℓ indolebutyric acid.

10. When roots have grown to approximately twice the size of the shoot (Fig. 49), remove plantlet from sterile culture and place in flask of full strength Hoagland solution covered to keep out light (Fig. 50). If roots stop growing on rooting medium, transfer to unsupplemented solid M medium. Keep plantlets in Hoagland's solution covered with polyethylene bags.
11. After 1 month transplant plants to potting medium (peat moss-vermiculite-perlite). Cover with polyethylene bag (Fig. 51). Fertilize monthly with liquid 20-20-10 mixture. Remove plastic bag after 2 weeks.
12. When white succulent buds and at least three sets of leaves have formed (Fig. 52), transplant to 1-quart containers to allow root growth (Fig. 53).
13. Transfer to nursery after about 1 month (Fig. 54) and when plants are large and sturdy plant in the field (Fig. 55).

The procedure for root sucker tip callus cannot be given as precisely. The tips are started in liquid or on solid M basal medium with the four auxins and five cytokinins. Callus or cell suspensions are also increased on this medium. Callus and cell suspensions of one clone have formed shoots after several subcultures to media containing high levels of dimethylallyladenine, other clones have not.

The successes so far achieved with the propagation of tissue from forest-grown trees--the hundreds of shoots that have been formed both in liquid and on agar media--suggest that complete success in propagating these trees will soon be possible by this method.

Figure 49. Root system about twice as large as top, and ready for placing in Hoagland's solution.

Figure 50. Plants held in flasks of Hoagland's solution wrapped in foil and covered with polyethylene bags to reduce transpiration.

Figure 51. Plants in tubes of potting mix inside polyethylene bags.

Figure 52. Plants in tubes, some now large enough for transplanting.

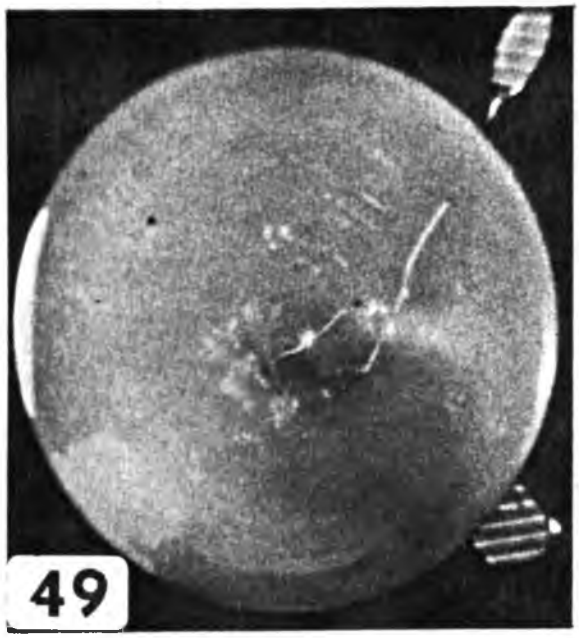
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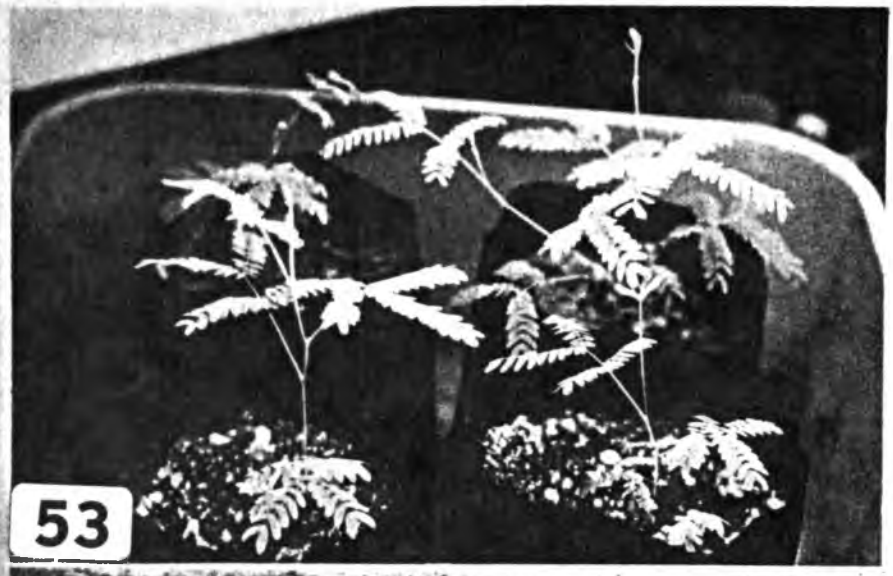
Figure 53. Plants in 1 quart plastic bags of potting medium growing rapidly.

Figure 54. Hardened trees in the nursery ready to go to the field.

Figure 55. A koa tree grown by tissue culture newly planted in the Laupahoehoe Forest Reserve.



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CHAPTER 4

VEGETATIVE PROPAGATION OF *ACACIA KOA* BY CONVENTIONAL METHODS

Introduction

Many conventional propagation techniques were tried as methods to clone selected forest-grown *Acacia koa* trees. These techniques included aseptic organ culture, which is closely related to tissue culture, grafting, air layering, and mist rooting. The studies also included attempts to induce root suckering in order to obtain juvenile material required for all types of propagation.

As was mentioned in the general introduction, very little vegetative propagation of koa had been done prior to the work reported here, so that almost nothing was known about its responses to various methods. It was not even known if the tree would form callus when wounded. The tree is easily propagated from seed so no vegetative propagation techniques had been tried. Therefore, all techniques tried, whether successful or not, are presented here.

The objectives of the experiments reported here were to develop techniques of conventional vegetative propagation that could be applied to large forest-grown koa trees.

Literature Review

It is common knowledge that most woody perennials propagate best when in the juvenile stage of development (Kormanik and Brown 1974). Foresters wishing to propagate mature trees, frequently resort to wounding, girdling, or cutting back in order to induce adventitious shoots to form so that they can be used for propagative material. These methods are used with *Liriodendron tulipifera* L. (McAlpine 1964),

Liquidambar styraciflua L. (Brown and McAlpine 1964), and *Nyssa sylvatica* Marsh. (Kormanik and Brown 1974), as well as many other hardwoods. Although some tree species can be vegetatively propagated using material from almost any part of the plant, it is always most likely that organs or plant parts that are immature, or at least, non-flower bearing, will be most likely to reproduce asexually (Hartmann and Kester 1975 p. 184-187).

Many plants produce different types of foliage on their juvenile parts than on their more mature parts. Generally, single leaf forms occur in the juvenile stage and more complex forms in the mature stage, as for example with *Citrus* spp., *Pinus* spp., and *Pisum* spp. (Leopold and Kriedemann 1974 p. 251). This is not the case with certain acacias such as *Acacia koa*. These trees produce compound leaves as their juvenile foliage and more simply shaped phyllodes as their mature foliage. Hartmann and Kester (1975 p. 184) illustrate this for the closely related *Acacia melanoxylon* R. Br.

The causes and control of juvenility in plants are not presently understood (Hartmann and Kester 1975 p. 184, Leopold and Kriedemann 1975, p. 249). However, much more is known about the influences of juvenility on rooting of cuttings, the location of juvenility in the plant, and about means of inducing juvenility. Pilet (1958) found higher levels of endogenous auxin in juvenile than in older pea tissues, suggesting a reason for the better rooting characteristics of juvenile cuttings. It was shown by de Muckadell (1954) that juvenility was located near the base in several tree species. Cuttings from the base produced propagules with juvenile characteristics, such

as thorns in locust, while those from the mature apex produced mature propagules. Juvenility can be modified, however, as has been found by de Muckadell (1956) and Stokes and Verkerk (1951), who grafted juvenile tissue to mature tissue and caused early flowering or maturity of the formerly juvenile tissue. Besides grafting, applications of gibberellins (Robbins 1957, Wadhi and Ram 1967) have caused reversion of mature to juvenile tissue and vice versa in different plants. Auxins also speed the conversion from juvenility to maturity (Zeeuw and Leopold 1955). Recent tissue culture experiments have demonstrated reversion from leaf tissue to juvenile buds in pines (Brown and Sommer 1977), Douglas-fir (Cheng 1975), and begonia (Doerr and Haramaki 1976) by the application of cytokinins. Although not a large change in reversing the onset of maturity, the change from leaf to bud tissue does suggest cytokinins may play a role in inducing juvenility.

The location of juvenility has been demonstrated for another acacia, *Acacia mearnsii* De Wild by Zeijlemaker (1976) who found that cuttings from the lower branches, where juvenility remained, rooted, whereas those from higher in the trees did not. His rooting system consisted of storing the cuttings for 2 weeks in Hoagland's solution in order to supply them a high level of nutrients. They were then placed under mist after treatment with rooting hormones. This acacia does not exhibit the difference between juvenile and mature foliage that koa does and it is considered difficult to root.

The techniques of aseptic organ culture have been well described by Murashige et al. (1972) and Murashige (1974a). They consist of

shoot tip culture, the rooting of shoot tips on aseptic media, shoot apical meristem culture, the formation of multiple shoots from just below the shoot apex, and shoot tip grafting *in vitro*. The most successful of these, according to Murashige (1974a), is shoot tip culture, but only with plants that are easily rooted from cuttings. Apical meristem culture and shoot tip grafting require so much skill and manual dexterity to perform that they are almost impractical (Murashige 1974a).

One of the requirements for successful conventional grafting is that the species being grafted form callus when it is wounded (Hartmann and Kester 1975, p. 374). Koa does not do this (author's observation). Other requirements are that the cambial regions be brought in intimate contact, and that the grafting operation be done when the stock and scion are in the proper physiological condition (Hartmann and Kester 1975). Assuming that this means at the time when bark "slips" easily in removal, such a proper physiological time was not found for koa (author's observation).

Air-layering has not been used much in forest tree propagation because it is such a slow procedure and the tree crowns are so difficult to reach (Sweet 1964). Despite this, it has been used occasionally to clone superior trees with considerable success by working small grafted trees (Barnes 1974). It has also been used to propagate sugar maple (Cunningham and Peterson 1965). It has come into increasing use as the very serious problem of graft incompatibility has come to be recognized in forest tree seed orchards around the world (Libby 1974). Air-layering is slow, sometimes taking

as long as two seasons with holly and magnolia (Creech 1954). However, air-layering can be used to root material that is more mature than that used for cuttings or grafts (Hartmann and Kester 1975).

Rooting of cuttings under mist is now the most common method of vegetatively propagating hardwood forest trees (Kormanik and Brown 1974). This method requires that the cuttings be physiologically juvenile (Kormanik and Brown 1974), that they be transported without drying out the cut ends (Libby 1974), and that they be kept in a mist bed with a temperature between 20° and 28° C (Kormanik and Brown 1974). These are the primary considerations. Other factors important to rooting are the clone and its inherent rooting potential (Filho and Yonezawa 1974), the use of rooting substances (Haissig 1974), the mist cycle (Hartmann and Kester 1975), and the aftercare given the propagule (Libby 1974).

Rooting of cuttings can be improved if the cuttings are supplied a source of nutrients. The work of Zeijelmaker (1976) in this regard has already been mentioned. Schreiber (1973) reported a highly significant increase in rooting of elm cuttings in simple hydroponic media. Root initiation requires energy, which is usually supplied by starch stored in the cutting (Haissig 1974). Mineral supplements can aid carbohydrate production in cuttings even while under mist (Preston et al. 1953).

A large amount of work has been done with various growth regulators in attempts to improve rooting of cuttings. Auxins such as IAA, NAA, and IBA are quite satisfactory for increasing root formation for those plants that produce abundant root primordia (Haissig 1974),

but auxins do not cause the primordia to form. Some other regulators are involved (Haissig 1974). Work on the inducement of root primordia in cuttings of difficult-to-root species has been much like that done in tissue culture meristemoid induction. With some plants, inhibition of IAA-oxidase with phenols has improved primordia formation (Jones 1976). With others, ethylene supplied by ethrel has given great improvement (Swanson 1976). Captan, a fungicide, has also shown an ability to induce root primordia in some species (Nanda et al. 1974). Just recently, Lee et al. (1977), found a significant increase in root primordia induction by pretreatment of the stems with acid or alkaline solutions. Acid loving plants rooted better after alkaline treatment and base loving plants after acid treatment.

Certain trees such as *Populus tremula* and *Malus* spp. can be propagated from root cuttings (Hartmann and Kester 1975). Since koa had been observed to root sucker, it seemed likely that it could also be propagated from root cuttings. Kormanik and Brown (1967), working with *Liquidambar stryaciflua*, found that shoots arose from special dormant shoot buds in the roots which could be induced to grow by additions of benzyladenine and gibberellins. Koa roots had been observed to sprout when given increased insolation (Spatz 1973), or when damaged (Baldwin and Fagerlund 1943). Pike (1972) suggested the use of many techniques including wounding, girdling, growth regulators, and temperature changes to induce sprouting from roots and root cuttings.

Related to juvenility and its location within the plant is a phenomenon called topophysis (Hartmann and Kester 1975, p. 185).

Plants that exhibit strong topophysis retain juvenile characteristics if propagated from juvenile plant parts and mature characteristics if propagated from mature plant parts. Two examples of this are *Araucaria* spp. and *Coffea* spp. Lateral branch cuttings from the lower parts of these trees will always grow in a horizontal, "branch-like" habit and, in *Araucaria* will only produce male strobili. In topophysis, the juvenile-to-adult ontogenetic phase changes are arrested. Such lack of change, if induced in koa by vegetative propagation, would prevent the use of vegetative propagation for tree improvement work.

Materials and Methods

The media and techniques used for aseptic organ culture were similar to those used for callus and cell suspension culture. Shoot tips of laboratory-grown seedlings and field-collected root suckers were surface sterilized and inoculated on agar nutrient media containing growth regulators intended to cause proliferation of buds, elongation of the shoot, or formation of roots directly from the shoot tip. The basal medium used was that of Murashige and Skoog (1962) and the growth regulators were; for proliferation and elongation: coconut milk, benzyladenine and dimethylallyladenine; for rooting: indoleacetic, indolebutyric, and napthaleneacetic acid.

The other aseptic organ culture technique tried was shoot tip grafting. For this, laboratory-grown seedlings that had just formed a shoot above the cotyledons were decapitated and inoculated to basal medium. Surface sterilized shoot tips of other seedlings and root sucker tips were then fixed to the stem ends with a drop of sterile lanolin. The same laboratory conditions were used for these cultures

for those discussed in previous chapters.

Grafting experiments were done by five different people, three of whom were skilled nurserymen. Bench grafting was done using 3- to 12-month-old pot-grown koa seedlings as root stocks. Grafted scions were from numerous sources and ontogenetic stages. The treatments are described in the Results Section. Some field grafting was done in the forest on the island of Hawaii. The techniques used were as described by Hartmann and Kester (1975). In most grafts, polyethylene film was used as the wrapping without grafting wax. All implements and areas to be wounded were washed with absolute alcohol just before working them.

Air layering experiments were made using polyethylene wrappers and, at first, two different media, a peat moss, vermiculite mixture or sphagnum moss. Root suckers, adventitious shoots, and branches were air-layered. Most of these were in the juvenile stage, although some were partly, or entirely in the mature foliage stage. Juvenile material was that with true leaves and mature material that with phyllodes. Girdles 1 to 2 cm long were made and 3000 mg/kg indolebutyric acid in talc was applied to induce rooting. The experiments were done on forest-grown trees at several locations on Oahu and Hawaii.

Experiments of propagation from root cuttings consisted primarily of wounding and chemical treatments of *in situ* roots in attempts to cause them to form suckers. The treatments are described in the Results Section. Root cuttings were also placed in flats and under mist following standard techniques as described by Hartmann and Kester

(1975).

Experiments on rooting of cuttings under mist were carried out in four different mist rooms or chambers. These were located in greenhouses at Waimanalo, Oahu, the upper Manoa campus of the University of Hawaii, at Hilo, Hawaii, and in an indoor chamber in a laboratory building. All facilities except the indoor chamber lacked light, temperature, and bottom heating controls. Mist was supplied intermittently at rates that would keep the leaves wet without dripping.

The rooting medium used most was perlite because more rooting occurred with it than any other medium. In some experiments hydroponic solutions were combined with the perlite which then served solely as a support medium. Small styrofoam cups were found to be ideal as pots for the rooting media, and provided isolation of treatments.

The other variables examined were the effects on rooting of various kinds, concentrations, and application techniques of auxins, ontogenetic stage of cuttings, mist cycles, bottom heat, and season. Unfortunately, the lack of suitable cuttings throughout the year prevented sufficient replication in time to determine the effects of season.

Cuttings ranged from 3 cm to 20 cm tall and from very succulent root suckers to hardened phyllodinous branch tips. Root sucker cuttings were quite variable in size and hardness because they were not found in sufficient quantities to provide opportunities for selection.

Results

Organ Culture

Attempts to induce rooting of shoot tips in aseptic media were generally unsuccessful. Roots were obtained on only three of numerous attempts at rooting of root sucker shoot tips. All three were on Murashige and Skoog's basal medium, one with 2.5 mg/ℓ 2,4-D and 10 percent coconut milk, one with the regulators of medium 30 (Table 12), and one with 15 mg/ℓ of dimethylallyladenine. These all eventually died, but they indicated that roots could be induced directly from shoot tips.

Three trials were made of shoot tip culture. In the first, laboratory-grown seedling shoot tips were inoculated on Murashige and Skoog's basal media containing no additives, 10 percent coconut milk, 1 mg/ℓ IBA, or 2 mg/ℓ NAA. All treatments were replicated three times. None of the tips became contaminated in this experiment, but all eventually died in culture.

The second experiment duplicated the media and replications of the first, but root sucker tips were used. All became contaminated and were discarded. The root sucker tips had been surface sterilized following normal tissue culture procedures, but like all other field-collected material, contained endogenous bacteria.

The third experiment was intended to induce shoot proliferation and elongation of root sucker shoot tips. Root sucker tips were placed on agar basal media containing 1 mg/ℓ benzyladenine (B), 5 mg/ℓ B, 1 mg/ℓ dimethylallyladenine (D), and 15 mg/ℓ D. One replication of 5 mg/ℓ B, one of 1 mg/ℓ D, and one of 15 mg/ℓ D were surface sterile,

the rest were contaminated. No growth of the surface sterile explants occurred.

Two trials were made of grafting shoot tips to decapitated seedlings aseptically. In the first trial, laboratory-grown seedling tips were used as the scions, but were all dislodged by movement of the stock plant hypocotyls, which were still elongating. In the second, root sucker tips were used. All but one of these were lost because of contamination. One remained sterile, but the tip died. However, multiple shoots grew from the decapitated seedling (Fig. 56). The development of multiple shoots suggested that decapitation followed by aseptic culture might offer propagation potential. However, this was not pursued.

Grafting

Grafting trials were conducted using several different materials. Scions were of both the true-leaf ontogenetic stage and the mature stage (Fig. 57). For most trials, polyethylene wrappings were used. The trials not made with polyethylene wrappings were numbers 6, 8, 9, and 11 to 15 (Table 14). In all, 202 grafts were attempted.

Some interesting observations of koa growth and development were made on young pot-grown seedlings used as rootstocks and placed under mist in the Waimanalo greenhouse. After 3 to 4 months in the very warm, very humid mist house, the rootstocks developed peculiarly shaped phyllodes over 30 cm long and only 1.5 cm wide (Fig. 58). These were similar to those formed later in the same greenhouse by the four tissue culture propagules of Figure 56. When some of these potted trees were moved from the mist house back to a normal

Figure 56. Two shoots which formed on seedling decapitated for aseptic grafting.

Figure 57. Graft of a phyllodinous tip.

Figure 58. Long, narrow phyllodes formed in a warm, wet mist room environment.

Figure 59. Trees with long, narrow phyllodes reverted to true-leaf stage and normal phyllodes when placed in normal climate at outdoor nursery.


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TABLE 14

GRAFTING TRIALS OF *ACACIA KOA*

Reference number	Number of grafts	Type of graft[a]	Scions[b]	Stocks	Remarks
1	36	Side veneer	Shoot tip (juv. & mature)	Seedling 4 mo. old	18 (9 of each scion) kept under mist
2	30	Side veneer	Root sucker tip (juv.)	Seedling 5 mo. old	One-half kept under mist
3	29	t-buds	Root sucker lateral (juv.)	Seedling 5 mo. old	One-half kept under mist
4	12	Side veneer	Stem sprouts (juv.)	Seedling 6 mo. old	Sprouts girdled, graft at swell above girdle
5	6	t-buds	Root sucker lateral (juv.)	Seedling 7 mo. old	Raffia and grafting wax, expert grafter
6	6	Side veneer	Root sucker tip (juv.)	Seedling 7 mo. old	Raffia and grafting wax, expert grafter
7	7	Top cleft	Root sucker tip (juv.)	Seedling 8 mo. old	
8	15	Patch buds	Root sucker lateral (juv.)	Seedling 9 mo. old	
9	15	Side veneer	Root sucker tip (juv.)	Seedling 9 mo. old	
10	6	Side veneer	Seedling branch (10 mo. old)	Seedling 10 mo. old	Scion and stock same plant
11	10	Top cleft	Seedling tip (juv.)	Forest trees to 1 yr. old	Seedling tips to forest-grown tree stumps
12	4	Approach	Root sucker propagule	Seedling 5 mo. old	Scions were potted cuttings and air layers
13	8	Side tongue	Seedling tip (juv.)	Seedling 7 mo. old	
14	10	Whip	Seedling tip (mature)	Seedling 10 mo. old	
15	8	Side veneer	Root sucker tip (juv.)	Seedling 10 mo. old	

[a] Descriptive terms are after Hartmann and Kester 1975.

[b] Juv. indicates juvenile or true-leaf stage; mature indicates phyllode stage.

environment at an outdoor nursery, they reverted first to the true-leaf stage (Fig. 59), and later to normal short, wide phyllodes. In the meantime, rootstocks kept continuously at the outdoor nursery remained in the true-leaf stage, eventually forming phyllodes at about the same time as the normal phyllodes were forming on the trees that had been under mist. It was apparent that the formation and shape of phyllodes were strongly influenced by temperature and perhaps humidity.

Another interesting observation made on these rootstocks was that under mist, 11 of 18 of them formed aerial roots from the two nodes immediately above the soil line. This suggests that the species has evolved the ability to root in sediments laid down by flooding as do many other trees.

After nine completely unsuccessful grafting trials, attempt number 10 (Table 14) was made. This was an attempt to graft seedling cuttings back onto the seedlings they had come from. Even this failed.

Two of the cleft grafts (No. 11, Table 14) made to the stumps of young trees in the forest survived for over a month, and one of the four approach grafts (No. 12) appeared for a time to have taken. These were the only near successes obtained. Basically, the difficulty seemed to be that callus tissue did not form on the wounded surfaces. Although all grafting experiments failed, they were reviewed here briefly so that they need not be repeated.

Air Layering

All the initial experimentation with air layering was done on

Oahu, working with phyllodinous laterals, partially phyllodinous adventitious shoots (Fig. 60), true-leaf shoots, and root suckers. A total of 39 trees and 24 root suckers were worked with. On these, at a total of 27 air layers were made on phyllodinous laterals or tops, 26 on partly true-leaf partly phyllodinous material, and 37 on true-leaf material.

The first tests were made using a mixture of vermiculite and peat moss as the rooting medium. It was not until sphagnum moss was used that successes began to be achieved. These successes were all with true-leaf root suckers. Two of the first group of successful air layers are shown in Figure 61. These rooted after 2 months. In all, 5 of the 24 air layers made on Oahu true-leaf root suckers rooted. Based on this evidence, the technique was immediately applied to superior tree root suckers on the island of Hawaii.

Of the 38 superior trees on Hawaii, 16 have produced root suckers and four have produced adventitious stem sprouts. Some produced abundant root suckers and some only one. A listing of all air layers made on superior tree root suckers and stem sprouts (Table 15), illustrates this variation. Trees 1, 14, and 36 have only been air layered once, because only one root sucker was available, but tree number 15 produced sufficient root suckers that 57 could be air layered.

Typically, the root suckers are only about 30 cm tall so that air layers were usually near the ground (Fig. 62). In this position they were frequently destroyed by feral pigs seeking the moisture inside the wrapper. This is one reason for the low level of success

Figure 60. Air layer of an adventitious shoot with terminal phyllodes.

Figure 61. Rooted air-layered root suckers from Aiea Heights (left) and Kalihi Valley (right).

Figure 62. Air-layered root sucker of tree number 5.


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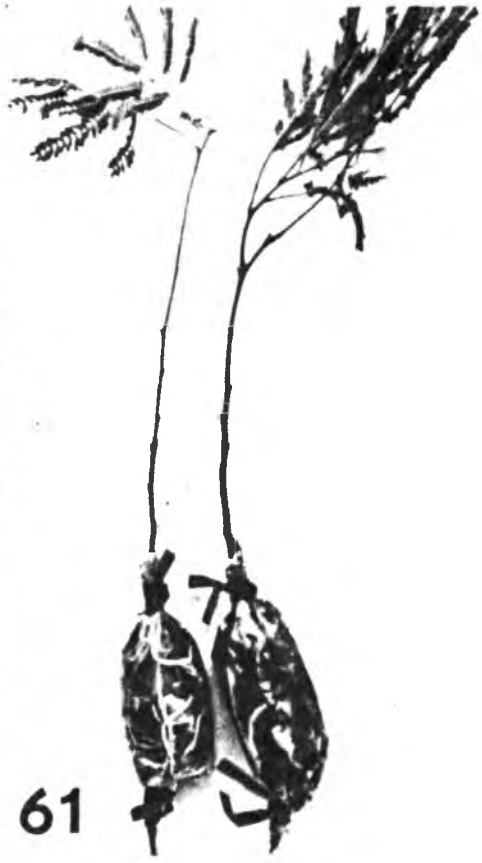
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TABLE 15.--AIR LAYERED ROOT SUCKERS AND STEM SPROUTS OF SUPERIOR TREES NUMBER AND SUCCESS

Tree number	Number of air layers	Number rooted	Number surviving
1	1	0	0
3	13	2	1
5	18	3	2
8	32	6	4
9	6	2	1
10	2	0	0
12	7	1	0
14	1	0	0
15	57	9	5
19	6	0	0
20	2	0	0
22	3	1	0
23	5	0	0
26	2	1	0
35	3	0	0
36	1	0	0
Total	159	25	13

indicated in Table 15. Another reason was that many air layers were made on root suckers that were too succulent. It was found after a long period that the only successes were occurring with air layers made on stems that had begun to form hard, brown-colored bark. In plant propagator's terms, this would be 6- to 8-month-old "wood."

Although the success in rooting was only 16 percent, the success in aftercare, that is, successful growth of the air-layered propagules, was a quite high 52 percent. The best technique of aftercare was found to be potting of the air layer immediately in a medium made of equal parts by volume of perlite, vermiculite, and peat moss. The air layer was kept under mist, with the rooting medium covered, for 2 to 3 weeks before placement in a lath house.

The 13 surviving air-layered propagules have been planted in the field in a small clonal progeny test. Most have produced the phyllode stage and one from tree number 3 is growing rapidly in normal form (Fig. 63). Others (Fig. 64) do not yet exhibit normal apical dominance, which may be a sign of topophysis. Two trees have not yet produced phyllodes, another possible sign of topophysis, or impaired maturation. The propagules tend to be susceptible to a rust disease, *Uromyces koae*, which is retarding their development.

Propagation from Roots

Since koa reproduces naturally from root suckers, a number of attempts were made to propagate it from root cuttings, or at least to induce suckering so that the root suckers could be rooted by other means.

Experiments with root cuttings, which were all unsuccessful,

Figure 63. Normal tree grown from an air-layered root sucker of tree number 3.

Figure 64. Air layer of tree number 5 exhibiting topophysis in growing as a branch rather than asserting apical dominance.

Figure 65. Root raised on a rock to alter growth regulator movement.

Figure 66. Girdled root forming new roots at distal side of girdle.


63


64

consisted of attempts to obtain shoots and roots from 68 root cuttings. Eighteen of these cuttings, which were all 8 to 10 cm long and of varying diameters, had existing suckers in place. Twenty-five root cuttings were kept for over 2 years in a nursery flat of vermiculite and peat moss without any sign of sprouting or growth. Forty-three others, with and without suckers, were kept under mist in perlite media. No rooting substances were used in these tests.

An extensive effort was made to induce suckering of koa roots *in situ*. In this work, 65 trees were selected on Oahu at various locations. The selection criterion was that the tree selected have exposed roots that could be worked.

Numerous treatments were applied to the roots, some of which were intended to simulate animal chewing, a suggested cause of suckering (Mueller-Dombois 1967). Other treatments included the application of chemicals likely to induce shoots or roots (Table 16). Knife wounds were made either down into the xylem (deep), or just down to the cambium (shallow). Roots were exposed to the sun by digging away soil covering them and cutting away the shading overstory. Raising of roots on rocks (treatment 8, Fig. 65) was tried because it was observed in the field that most natural root suckers occurred where roots grew over rocks. Possible explanations are the alteration of growth regulator movement or the large temperature variations of the exposed rock surface. The chemicals of treatments 10 to 16 were swabbed onto and in the vicinity of knife wounded areas. Treatments 17 and 18 consisted of wrapping girdled roots in moist sphagnum moss and wrapping with polyethylene film as in air layering. This was

TABLE 16.--TREATMENTS APPLIED TO *ACACIA KOA* ROOTS INTENDED TO INDUCE SUCKERING

Number	Treatment	Number of trees	Number of treatments
1	Knife wounding (deep)	65	195
2	Knife wounding (shallow)	65	195
3	Expose root to sun	65	65
4	"Chew" with pliers	20	40
5	Pound with hammer	20	40
6	Heat with torch	20	40
7	Girdle root	32	32
8	Raise root on rock	9	18
9	Bury exposed root	11	11
10	Wound - kinetin (100 mg/ℓ)	31	48
11	Wound - NAA (500 mg/ℓ)	27	35
13	Wound - Ethrel (100 mg/ℓ)	15	17
14	Wound - B (500 mg/ℓ)	31	48
15	Wound - IAA (200 mg/ℓ)	15	17
16	Wound - GA (500 mg/ℓ)	31	48
17	Air layer (untreated)	6	6
18	Air layer B (500 mg/ℓ)	6	6

tried after shoots had been found to form under the moss in two root sucker air layers. The growth regulators were tried on the basis of observations in the literature (Pike 1972, Kormanik and Brown 1967) that had shown benzyladenine, idoleacetic acid, and ethrel to induce bud formation or rooting.

The only effect any of these treatments had, other than slight swelling above some of the girdles, was the development of a new root system at the distal side of one girdled root (Fig. 66) located in a humid forest in Nuuanu Valley. Although koa produces root suckers quite readily both in savanna and dense rain forest situations, the mechanism by which it does so, and methods that will induce suckers to develop, remain unknown.

Rooting Under Mist

The first experiment in rooting under mist (Nos. 1, 2, 3, Table 17) was a comparison of two methods of auxin application with or without bottom heat, on succulent phyllodinous tip cuttings in a vermiculite-peat moss medium. It also included untreated controls (No. 3, Table 17). All the cuttings died rapidly of fungus contamination indicating the medium was a poor choice.

The next experiment (Nos. 4, 5, Table 17), was a comparison of true-leaf and phyllodinous cuttings obtained from adventitious stem and branch sprouts. Perlite, which was used as the medium, functioned well. None of the phyllodinous cuttings rooted, but nine of the true-leaf cuttings did. Only four or five roots developed on each cutting (Fig. 67), and all of them died after transplanting into a potting medium.

TABLE 17

MIST ROOTING EXPERIMENTS CONDUCTED ON *ACACIA KOA* CUTTINGS

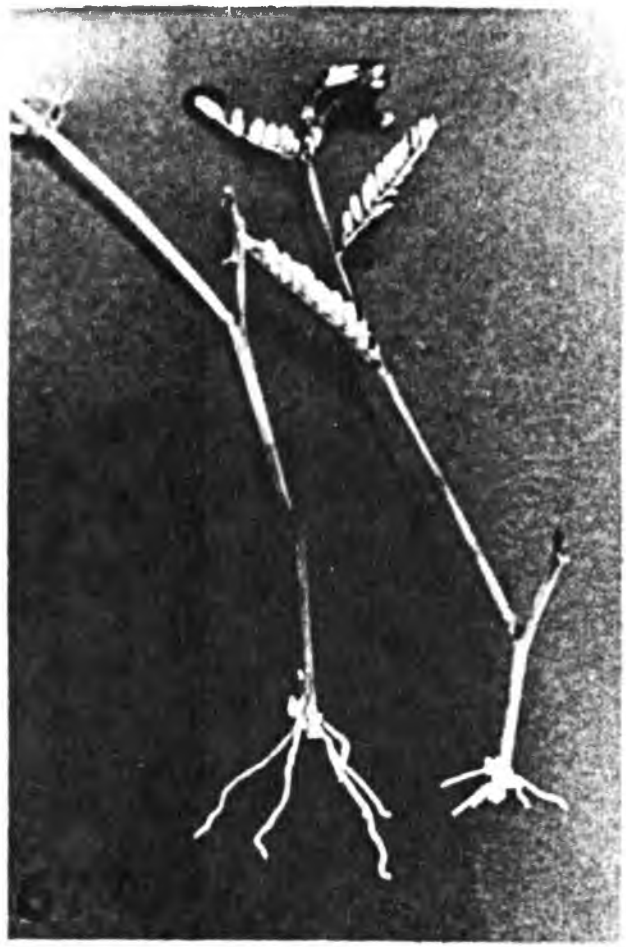
Ref. no.	Number cuttings	Ontogenetic stage	Source	Auxin	Auxin strength	Application method	Rooting medium	Number rooted	Remarks
1	50	Succulent phyllode	Large trees	IBA	3000 mg/kg	Talc	1:1 vermiculite-perlite	0	One-half with bottom heat
2	50	Succulent phyllode	Large trees	IBA	100 mg/ℓ	1 hr soak	1:1 vermiculite-perlite	0	One-half with bottom heat
3	50	Succulent phyllode	Large trees	None	-	-	1:1 vermiculite-perlite	0	One-half with bottom heat
4	25	Phyllode	Stem sprouts	IBA	3000 mg/kg	Talc	Perlite	0	-
5	25	True-leaf	Stem sprouts	IBA	3000 mg/kg	Talc	Perlite	9	36% rooted
6	54	True-leaf and Phyllode	Root suckers	IBA	3000 mg/kg	Talc	Perlite	8	15% of true-leaf rooted
7	20	True-leaf and Phyllode	Root suckers	IBA	10 mg/ℓ	In medium	0.002 M KNO_3 and perlite	6	50% true-leaf, 1% phyllode rooted
8	20	True-leaf and Phyllode	Root suckers	IBA	10 mg/ℓ	In medium	0.002 M KH_2PO_4 and perlite	0	-
9	20	True-leaf and Phyllode	Root suckers	IBA	10 mg/ℓ	In medium	Hoagland's solution and perlite	3	30% of true-leaf rooted
10	10	True-leaf and Phyllode	Root suckers	None	-	-	Water and perlite	0	-
11	30	True-leaf	Root suckers	IBA	3000 mg/kg	Talc	Perlite	10	30% rooted
12	10	True-leaf	Root suckers	NAA	2000 mg/kg	Talc	Perlite	0	-
13	62	True-leaf	Root suckers	IBA	3000 mg/kg	Talc	Perlite	7	11% rooted

TABLE 17. (Continued) MIST ROOTING EXPERIMENTS CONDUCTED ON *ACACIA KOA* CUTTINGS

Ref. no.	Number cuttings	Ontogenetic stage	Source	Auxin	Auxin strength	Application method	Rooting medium	Number rooted	Remarks
14	37	True-leaf	Root suckers	IBA	3000 mg/kg	Talc	Perlite	10	27% rooted
15	9	True-leaf	Stem sprouts	IBA	1000 mg/kg	Talc	Running water	0	-
16	9	True-leaf	Stem sprouts	IBA	3000 mg/kg	Talc	Running water	0	-
17	9	True-leaf	Stem sprouts	IBA	8000 mg/kg	Talc	Running water	0	-
18	108	True-leaf	Root sucker	IBA	3000 mg/kg	Talc	Perlite	19	18% rooted
19	10	True-leaf	Root sucker	IBA	3000 mg/kg	Talc	Perlite after 10 min NaOH	3	-
20	10	True-leaf	Root sucker	IBA	3000 mg/kg	Talc	Perlite after 10 sec H_2SO_4	1	-
21	10	True-leaf	Root sucker	IBA	3000 mg/kg	Talc	Perlite	1	-
22	10	True-leaf	Stem sprouts	None	-	-	Perlite after 10 min NaOH	0	-
23	10	True-leaf	Stem sprouts	None	-	-	Perlite after 10 sec H_2SO_4	0	-
24	10	True-leaf	Stem sprouts	None	-	-	Perlite	0	-

Figure 67. Rooted true-leaf root sucker cuttings. Note the poor root systems which are typical of those formed.

Figure 68. Various koa cutting experiments in the mist room.



During the same period, a test of 54 true-leaf and phyllode cuttings from superior tree root suckers was carried out. The mist bench was quite cluttered (Fig. 68), because roots did not form for 2 to 4 months. Eight of the true-leaf cuttings rooted, but none of the phyllodinous cuttings did. These cuttings were transplanted into a mixture of equal parts by volume of vermiculite, peat moss, and perlite and kept under mist, but again they all died. It was evident that the condition of the rooted cuttings was so poor after the lengthy period under mist, that their survival after transplanting was a major problem.

The next experiment was intended to accelerate rooting by supplying nutrients. Schreiber (1973) had obtained good rooting of elm cuttings with hydroponic solutions containing IBA as the rooting media, so his solutions were used (Nos. 7-10, Table 17). For this test, equal numbers of true-leaf phyllode root sucker cuttings were used. The three hydroponic solutions and water control were placed in styrofoam cups of perlite covered with aluminum foil. The cutting stems were then punched through the foil and placed under mist. The foil sealed the stems so that stem flow from the misting would not dilute the solutions. Despite this, leakage and dilution were variable among treatments. The rooting substance IBA was added directly to the hydroponic solutions except the water control.

The potassium nitrate solution gave the best rooting with one-half the true-leaf cuttings and one phyllode cutting forming roots. This was the only phyllode cutting ever to produce a root. No roots were produced in the monobasic potassium phosphate solution, but

30 percent rooting was obtained in Hoagland's solution. It was noted that stems turned brown and no rooting occurred in the cups that did not appear to leak, that is, those with the least diluted solutions. Possibly, the concentration of IBA at 10 mg/ℓ was too high and the dilution was beneficial. Dilution of nutrients may also have been beneficial. Hoagland's solution is often diluted for use as a nutrient medium.

Although rooting was improved in the hydroponic solutions, none of cuttings survived transplanting, even though the transplanted cuttings were held under mist. Two repeat experiments were made using potassium nitrate and Hoagland's solution and 10 true-leaf root sucker cuttings in each solution in one experiment and 13 similar cuttings per treatment in the other. No rooting was obtained. The rooting from the first test may have resulted because the cuttings were collected the same day the experiment was set up. The cuttings used in the other tests were 1 or 2 days old.

A comparison of 3000 mg/kg IBA in talc against 2000 mg/kg NAA as the rooting substance was established next (Nos. 11, 12, Table 17). In this test, 30 percent of the IBA treated cuttings rooted. These ten cuttings were transplanted into plastic tubes of potting mix, which were then covered with parafilm to prevent water entry. The plants were held under mist for 2 months at which time some had formed new apical buds. Five survived the period under mist, and four survived after being removed from the mist. These superior tree cuttings, the first to survive, are shown in Figure 69 along with a fifth cutting from a later experiment. They were transplanted from

the tubes to the 1-quart plastic bags shown.

It was by this time apparent that only cuttings in the true-leaf stage could be expected to root and that IBA in talc provided good success in rooting. The next group of cuttings tried (No. 13, Table 17) was 62 true-leaf root sucker cuttings. Only seven of these rooted. One of the seven survived transplanting and is the fifth plant in Figure 69. All rooting of superior tree root sucker cuttings was extremely variable, probably because the cuttings were very variable. By observation of many such cuttings, it was determined that those with the highest rooting frequency were the ones on which hard bark was just beginning to form.

In the next group (No. 14, Table 17) under the same conditions as the previous test, 27 percent rooted. Two of these survived transplanting, giving an overall survival to that point of 3.1 percent of the 224 true-leaf root sucker cuttings that had been tried up until that time. The average rooting percentage for all 224 cuttings was 19.2. Survival after transplanting was obviously the major problem.

Repeated failures occurred at a facility in Hilo, which proved to be too humid, and at another facility established in upper Manoa Valley, which had serious temperature and wind difficulties. Altogether, 13 experiments were ruined by equipment failure. Because of these and other problems, experiments were moved indoors into controlled laboratory conditions.

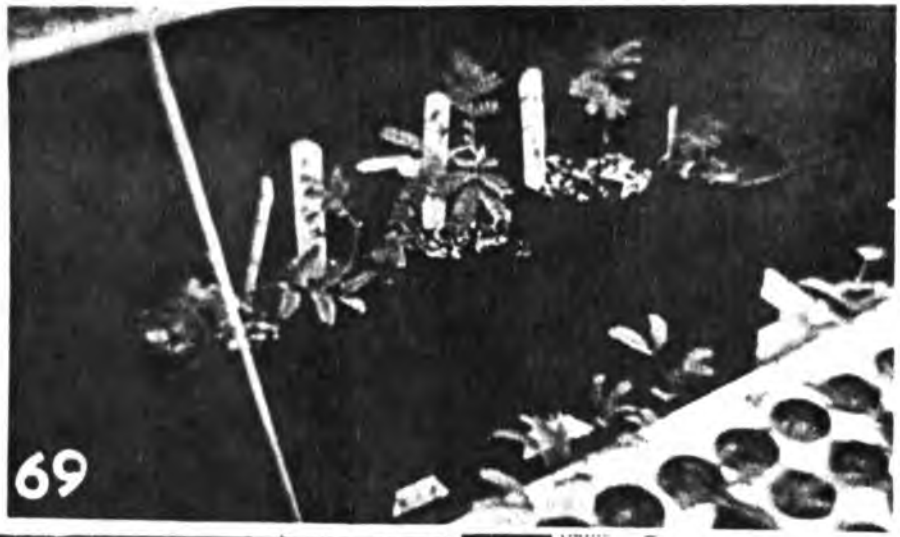
The first experiment established under laboratory conditions was a test of rooting in running water (Nos. 15, 17, Table 17). Three levels of IBA in talc were used on true-leaf cuttings from pot-grown

Figure 69. Five koa trees started from cuttings growing in plastic bags at Waimanalo nursery.

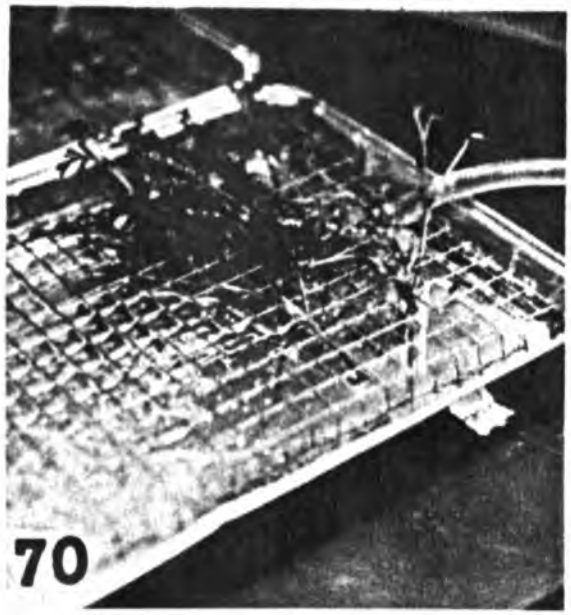
Figure 70. A rooting experiment using running water rather than mist.

Figure 71. Mist chamber constructed to overcome variables encountered in other mist facilities.

Figure 72. Koa trees started from air layers and cuttings of superior trees growing in an exclosure at the Laupahoehoe Forest Reserve.



69



70

71

72

stem sprouts. The cuttings were held in a wire frame so that water would flow over their stems (Fig. 70). Artificial light was supplied.

An indoor mist chamber was then constructed in which light, mist cycle, bottom heat, and sterility could be controlled (Fig. 71). Five groups of superior tree root sucker cuttings have been placed in this chamber for rooting. A total of 19 cuttings rooted out of 108 (No. 18, Table 17). The fourth group was treated with a combination of 3000 mg/kg IBA and 50 percent captan. These plants produced no roots at all, so the captan treatment was dropped. Light, originally at 42 $\mu E\ m^{-2}sec^{-1}$, was increased to 110 $\mu E\ m^{-2}sec^{-1}$ in the hope that improved rooting performance would be obtained.

The most recent experiments undertaken were based on Lee et al. (1977), who found that sodium hydroxide or sulfuric acid pretreatments to cutting stems aided rooting. In both experiments (Nos. 19-24, Table 17) the basal portion of the stems were treated with a 10-minute soak in pH 10.5 sodium hydroxide, a 10-second dip in 0.2 N sulfuric acid, or a 10-minute soak in distilled water (control). In one experiment, all cuttings were treated with 3000 mg/kg IBA. In the other, no auxin was used. The first experiment clearly indicated a benefit from the sodium hydroxide treatment (No. 19, Table 17). The second experiment, as compared with the first, indicated a requirement for the IBA treatment.

Seven of the trees grown from superior tree cuttings have been planted in the forest (Fig. 72). One, a propagule of tree 15, died during the first few months after planting. Three of the other six were found to be infested with the rust *Uromyces koae* which is

distorting their growth. All have formed phyllodes, but all so far appear much less vigorous than seedling trees growing in the same area.

Discussion

The air layering and mist rooting experiments clearly demonstrate that juvenility is essential for the successful propagation of koa as it is for many other trees. However, unlike some species, koa retains the potential to revert to juvenility in its upper branches as well as on its stem and its roots. The occasional rooting of adventitious stem sprout cuttings indicates that the true-leaf stage may be an indicator of juvenility in all parts of the tree, not just at its base as with many other trees.

The results of the grafting work show that koa is extremely difficult or perhaps impossible to graft. It could not even be grafted back to itself. Approach grafting appeared to be the only type that might be successful. Some method of inducing callus to form on wounded areas is needed, but there was no indication of callus formation on wounded roots after auxin or cytokinin application. The only swelling or abnormal growth induced was by girdling. It may be possible to graft previously girdled material.

The changes that occurred in the seedling trees used for rootstocks when they were moved into and out of moist-warm conditions suggest that considerable manipulation of ontogenetic stages should be possible with the species. This might be beneficial in providing juvenile material for propagation if plants were grown in cool, shaded locations to lengthen their period of juvenility.

Of the conventional propagation methods tried, the only successes were with air layering and mist rooting. With both, there was low survival of the rooted propagules after transplanting. This was much more of a problem with the mist rooted cuttings than with the air layers. The problem was that by the time they had produced roots the propagules had used up most of their stored nutrients, and most of their leaflets had abscised. Few roots were formed and those that did probably were non-functional. The roots required a supply of nutrients immediately after transplanting to become functional.

Perhaps the solution to this problem may be drawn from the results with tissue culture-grown plantlets. These produced functional roots when placed in Hoagland's solution.

The percentage of success in rooting air layers should increase now that experiments have shown that only root suckers with hardening stems provide suitable material.

Unfortunately, there is so little suitable juvenile material available from the superior trees either for air layering, or for mist rooting, that many years will go by before clones of any appreciable size can be developed, even if the percent of success is greatly improved.

Coupled with this, the propagules so far produced are, for the most part, not growing at all like the trees from which they came. Many are disease susceptible and others are poorly formed. For these reasons, although cloning of koa by conventional means has been shown to be possible, it is not at all certain that it is practical.

Conclusions

Acacia koa can be propagated by air layering or mist rooting of cuttings, if the propagative material is in the true-leaf ontogenetic stage and has bark just beginning to harden and brown.

Air layering successes were obtained on root suckers and stem sprouts using a girdle 2 to 3 cm long, 3000 mg/kg IBA in talc above the girdle, slightly moist sphagnum moss, and polyethylene wrappers. Air layers of root suckers were frequently damaged by pigs and require fencing.

Mist rooting was successful most frequently using a perlite medium, a mist cycle sufficient to keep the leaves moist without dripping, 3000 mg/kg IBA in talc at the base of the cutting, and cuttings with at least three leaves. Normal greenhouse sunlight resulted in the best rooting of cuttings.

Rooted propagules were difficult to keep alive after transplanting, even when kept under mist. The most successful procedure found has been to pot the propagule in peat moss-vermiculite-perlite, cover the medium to prevent water entry, and return the plant to mist until new buds form. This procedure has not yet proved highly successful, but has resulted in the survival of all the propagules so far produced. More experimentation in aftercare is needed.

Propagules produced by air layering had a much higher survival rate in aftercare than those grown from rooted cuttings. They also grew into better looking trees when planted in the forest. However, both types of propagules were inferior in form and disease resistance to natural seedlings, at least during the first 1 1/2 years of growth.

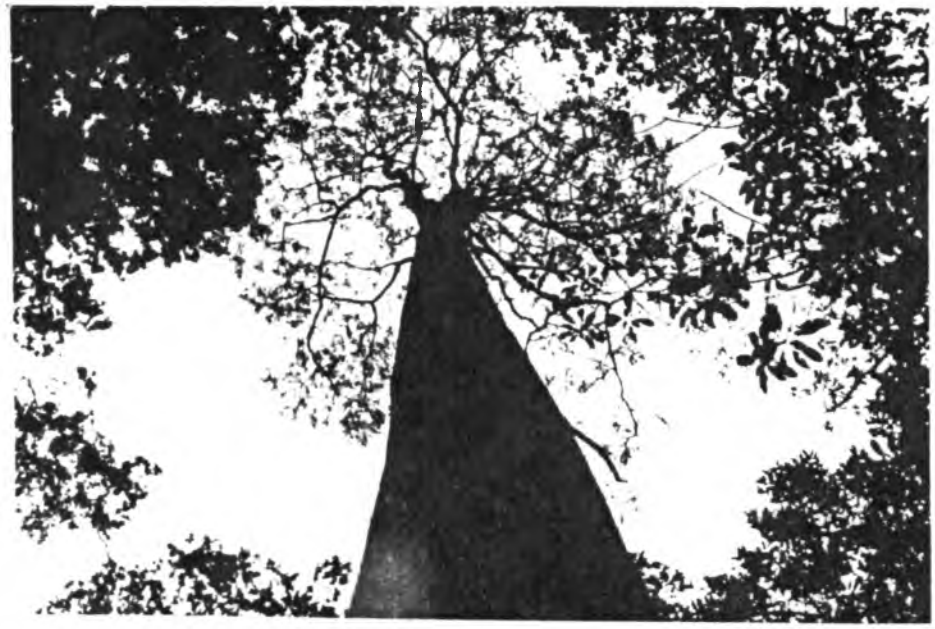
It was also possible that topophysis was present in some propagules.

Of the air layers attempted, 16 percent rooted and 52 percent of those (13 trees) survived aftercare to grow as trees. Among the cuttings from similar root sprout material, 19 percent rooted and 16 percent of those (7 trees) survived aftercare. Air layering must be recommended on the basis of better percentage until the survival of rooted cuttings can be improved.

Unfortunately, propagation of superior trees by air layering lacks practicality because only about one-half of the trees produce suckers, because air layering is a very slow method of increasing a clonal population, and because of difficulty of working the scattered, hard-to-reach trees on a regular basis.

Therefore, in summary of the entire paper, it can be concluded that mature trees of *Acacia koa* can be cloned, if "cloning" is accepted to include some phenotypic aberrations. Although only seedling tissue has been made to regenerate trees by tissue culture, there is evidence to suggest that tissue from mature trees will do so also. Superior trees have been propagated by air layering and mist rooting of cuttings and the propagules are now growing in the forest. Let us hope that some day, a propagation technique will be found that will result in a forest of giant koa trees such as those in Figure 73.

Figure 73. Superior koa trees, numbers 35 and 30. Tree number 30 has the straightest bole on any tree selected.



APPENDIX A

FORMULATIONS OF MURASHIGE AND SKOOG (1962) AND SCHENK AND HILDEBRANDT (1972) BASAL MEDIA

Chemical	M[a]	SH[b]
	mg/ℓ	
NH_4NO_3	1650	-
KNO_3	1900	2500
$NH_4H_2PO_4$	-	300
$CaCl_2 \cdot 2H_2O$	440	200
$MgSO_4 \cdot 7H_2O$	370	400
KH_2PO_4	170	-
Na_2 EDTA	37.3	37.3[c]
$Fe_2(SO_4)_3$	27.8	27.8[c]
$MnSO_4 \cdot 4H_2O$	16.9	10.0
H_3BO_3	6.2	5.0
$ZnSO_4 \cdot 7H_2O$	8.6	1.0
KI	0.83	1.0
$CuSO_4 \cdot 5H_2O$	0.025	0.2
$NaMoO_4 \cdot 2H_2O$	0.25	0.1
$CoCl_2 \cdot 6H_2O$	0.025	0.1
Thiamine	5.0	5.0
Nicotinic acid	5.0	5.0
Pyridoxine·HCl	0.5	0.5
Glycine	2.0	-
Myo-inositol	100[d]	1000[d]
Sucrose	30000[d]	30000[d]

[a] Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Phys. Plant. 15:473-497.

[b] Schenk, R. V. and A. C. Hildebrandt, 1972. Medium and technique for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. Jour. Bot. 50:199-204.

[c] Not as published.

[d] Varied in some experiments.

APPENDIX B

EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal[a] medium	Solid[b] or liquid	Growth regulators[c]														Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	SL															0	1	0
M	L														0.1	0	1	0
M	SL														1	0	1	0
M	SL														2	0	1	0
M	SL														5	0	1	0
M	SL														10	1	1	1
M	SL														15	1	1	0
M	SL														20	0	1	0
M	S														30	0	0	0
M	S														40	0	0	0
M	S	20														-	0	0
M	S	20													10	0	1	0
M	S	20				0.5									10	-	1	0
M	S	20				2.5									10	1	1	0
M	S	20					0.2								10	2	2	1
M	S	20					2.5								10	1	2	0
M	S	20					5								10	1	1	0
M	L	20								1						-	0	1
M	L	20								10						-	0	0
M	L	20								15						-	0	1
M	S		0.1													0	0	0
M	S		0.1												10	0	1	0
M	S		0.2													0	0	0
M	S		0.3													0	0	0
M	SL		2	2			2.5					2.5			-	1	0	
M	SL		2			2		2.5		2.5						-	2	2
M	L		2			2		2		3						-	2	2
M	L		2			2		4		6						-	2	2
M	SL		2		2			2.5		2.5						-	1	0
M	S			0.2												-	0	3[e]
M	S			0.3												-	0	3[e]
M	S			0.5												-	0	2[e]
M	S			2.5												-	0	0
M	S			2.5											5	-	0	0
M	S			2.5											10	-	1	0
M	S			2.5											20	-	1	0
M	S			5												-	0	0
M	S			5											5		2	0

APPENDIX E. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal medium[a]	Solid or liquid[b]	Growth regulators[c]													Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	S			5											10		3	1[e]
M	S			5											20		2	0
M	S			10												-	0	0
M	S			10											5	-	3	1
M	S			10											10	-	2	1
M	S			10											20	-	2	1
M	S			1		2									10	-	0	0
M	SL				5											0	1	0
M	SL				2.5			5								2	2	0
M	SL				5			5								2	2	0
M	SL				5			5			5					2	2	1
M	SL				5		0.2				5					-	2	1
M	SL				2		2	5								-	2	0
M	SL				4			5								-	2	0
M	L				0.5			1								-	1	0
M	L				5			2			5					-	2	0
M	SL				4			2.5		2.5						-	2	0
M	SL				2		2	2.5		2.5						-	2	0
M	S				0.5											-	1	0
M	S				1											-	1	0
M	S				2.5											-	1	0
M	SL				2	2		2.5					2.5			-	1	0
M	S				5			5							10	0	1	0
M	S					1.25		1								0	1	1[e]
M	S					1.25		5								0	0	0
M	S					1.25		10								0	0	0
M	S					2.5		1								0	1	1[e]
M	S					2.5		5								0	0	0
M	S					2.5		10								0	2	1[e]
M	S					5		1								0	0	0
M	S					5		5								0	0	0
M	S					5		0								0	0	0
M	S					0.1										-	0	0
M	S					0.5										-	0	1[e]
M	S					0.2									10	1	1	0
M	S					0.25										-	0	0
M	S					1										-	0	0
M	L					0.2				1.5						-	1	0
M	L					2	0.2	2		3						-	1	0

APPENDIX B. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal medium[a]	Solid or liquid[b]	Growth regulators[c]														Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	S					2	0.2	5		10						-	1	0
M	S					4				15						-	1	0
M	S					2.5									10	1	1	0
M	SL						0.2									0	0	0
M	S						0.2								1.25	0	1	0
M	S						0.2								2.5	0	1	0
M	S						0.2								5	0	1	0
M	S						0.2								10	2	2	1
M	S						0.4									0	0	0
M	S						0.4								1.25	0	1	0
M							0.4								2.5	0	1	0
M							0.4								5	0	1	0
M							0.4								10	0	1	0
M	S						0.8									0	1	0
M	S						0.8								1.25	0	1	0
M	S						0.8								2.5	1	3	0
M	S						0.8								5	1	2	0
M	S						0.8								10	1	3	0
M	S						1.6									0	1	0
M	S						1.6								1.25	1	1	0
M	S						1.6								2.5	1	2	0
M	S						1.6								5	1	2	0
M	S						1.6								10	1	2	0
M	SL						1.25								1	1	2	1
M	SL						2.5								1	1	2	1
M	SL						5.0								1	1	2	1
M	SL						1.25								5	1	2	1
M	SL						2.5								5	1	2	0
M	SL						5.0								5	1	2	0
M	SL						1.25								10	1	2	0
M	SL						2.5								10	2	2	0
M	SL						5.0								10	2	2	0
M	SL						1.25								15	1	2	0
M	SL						2.5								15	1	2	0
M	SL						5.0								15	1	2	0
M	SL						2.5								0.5	0	1	0
M	SL						0.2	5			5					0	2	0
M	L						0.2	0.2		0.2						-	2	1
M	L						0.2	0.5								-	2	1
M	L						0.2	0.1		0.1						-	1	1

APPENDIX B. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal medium[a]	Solid or liquid[b]	Growth regulators[c]													Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	L						0.5	0.5		0.5						-	1	0
M	L						0.5	0.2								-	1	0
M	L						0.2	0.2								-	1	0
M	SL						4	5								0	1	0
M	SL						0.25									-	0	0
M	SL						0.5									-	0	0
M	SL						1									-	0	0
M	SL						0.25	1								-	0	0
M	SL						0.5	1								-	0	0
M	SL						1	1								-	0	0
M	SL						0.25	5								-	1	0
M	SL						0.5	5								-	1	0
M	SL						1	5								-	1	0
M	SL						0.25	10								-	2	0
M	SL						0.5	10								-	1	0
M	SL						1	10								-	1	0
M	SL						0.2			5						-	1	0
M	SL						0.2			10						-	2	1
M	SL						0.2			15						-	3	2
M	SL							0.1								-	1	0
M	S							0.5								-	0	0
M	SL							1								-	1	1
M	S							2								-	0	0
M	S							4								-	1	0
M	SL							5								0	2	2
M	S							10								-	1	2
M	S							15								-	1	0
M	S							20								-	1	0
M	S							25								-	0	0
M	S	20						0.5								-	0	0
M	S	20						1								-	0	0
M	S	20						2								-	0	0
M	S	20						4								-	1	0
M	S							0.5			5					-	0	0
M	S							1			5					-	0	0
M	S							2			5					-	0	0
M	S							4			5					-	0	0
M	SL							5			5					0	1	1
M	SL							5			10					-	1	0
M	S							10			5					-	2	1
M	SL							1			1					-	0	0

APPENDIX B. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal[a] medium	Solid[b] or liquid	Growth regulators[c]													Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	L							0.1		0.1						-	1	0
M	L							1		1						-	1	1
M	SL							2		3						-	1	1
M	SL							5		10						-	2	3
M	SL							10		10						-	2	2
M	SL							10		20						-	1	1
M	S							5						10		-	1	0
M	S							2.5							1.25	-	1	0
M	S							5							2.5	-	3	0
M	S							10							5	-	1	0
M	S							2.5							1.25	-	1	0
M	S							5							2.5	-	1	0
M	S							10							5	-	1	0
M	S							2.5							1.25	-	1	0
M	S							5							2.5	-	1	0
M	S							10							5	-	1	0
M	S								5							-	0	0
M	S								10							-	0	0
M	S								20							-	0	0
M	S								30							-	0	0
M	SL									1						-	1	1
M	SL									5						-	1	0
M	SL									10						-	1	1
M	SL									15						-	3	3
M	S									20						-	1	0
M	S									15	5					-	0	0
M	S											1				-	0	0
M	S											2				-	0	0
M	S											4				-	0	0
M	SL												5			-	1	0
M	SL		1		1	1	1	1	1	1	1		1	2		3	3	2
M	SL		1		1	1	1	1	1	1	1		1	2	1	0	2	1
M	SL		1		1	1	1	1	1	1	1		1	2	10	-	1	0
M	SL		1		1	1	1	1	1	1	1		1			-	2	0
M	SL		1		1	1	1	1	1	1	1	1		2		-	2	0
M	SL							1	1	1	1		1	2		-	1	0
M	SL		1		1	1	1									0	0	0
M	SL							2	2	2	2		2			0	2	1
	SL							2	2	2	2		2		10	0	2	0
M	SL		1	1	1	1	1	1	1	1	1	1		2		1	1	0
M	SL		.02	.02	.02	.02	.02	.04	.04	.04	.04	.04				0	0	0

APPENDIX B. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal[a] medium	Solid[b] or liquid	Growth regulators[c]													Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/ℓ													%			
M	SL		.04	.04	.04	.04	.04	.02	.02	.02	.02	.02				0	0	0
M	S		.04	.04	.04	.04	.04	0.1	0.1	0.1	0.1	0.1				0	0	0
M	S		.04	.04	.04	.04	.04	0.2	0.2	0.2	0.2	0.2				0	0	0
M	S		0.1	0.1	0.1	0.1	0.1	1	1	1	1	1				0	1	0
M	SL		0.2	0.2	0.2	0.2	0.2	1	1	1	1	1		2		0	1	0
M	SL		0.6	0.6	0.6	0.6	0.6	1	1	1	1	1				1	3	0
M	SL		0.8	0.8	0.8	0.8	0.8	1	1	1	1	1				1	3	0
M	SL							0.2	0.2	0.2	0.2	0.2				0	0	0
M	SL							1	1	1	1	1				0	1	0
M	S		.05	.05	.05	.05	.05	.05	.05	.05	.05	.05				0	1	0
M	S		.05	.05	.05	.05	.05	1	1	1	1	1				0	1	0
M	S		.05	.05	.05	.05	.05	2	2	2	2	2				0	1	0
M	S		1	1	1	1	1	.05	.05	.05	.05	.05				0	0	0
M	S		1	1	1	1	1	2	2	2	2	2				0	1	0
M	S		2	2	2	2	2									0	0	0
M	S		2	2	2	2	2	.05	.05	.05	.05	.05				0	1	0
M	S		2	2	2	2	2	1	1	1	1	1				0	1	0
M	S		2	2	2	2	2	2	2	2	2	2				0	1	0
M	SL		1		1	1	1	1		1	1	1	1	2		2	2	0
M-	S	20					0.2								10	-	1	1
M-	S	20					2.5								10	-	2	0
SH	L															-	0	0
SH	SL					0.2										0	1	0
SH	SL					1					0.5					0	1	0
SH	SL					2.5					1					0	1	0
SH	SL										0.2					0	0	0
SH	L				2.0		0.2				0.1					2	2	1
SH	L				2.0		0.2				0.1				10	0	1	0
SH	L				2.0		0.2								10	0	1	0
SH	L						0.2				0.1					0	1	0
SH	L				4.0		0.2				0.1					0	1	0
SH	L							0.1								-	0	0
SH	L							1								-	1	0
SH	L				2.0		0.2	0.1								-	0	0
SH	L				2.0		0.2	1								-	1	0
SH	L	20			2.0		0.2				0.1					0	1	0
SH	L	20			5.0		0.2				0.1					0	1	0
SH	L							2								-	0	0
SH	L							5								-	0	0
SH	L				2.0		0.2	0.5								-	1	0
SH	L						0.2	0.1			0.1					-	1	0

APPENDIX B. (Continued) EFFECTIVENESS OF BASAL MEDIA AND GROWTH REGULATOR SUPPLEMENTS GIVEN TRIAL FOR CALLUS INDUCTION, CALLUS AND CELL SUSPENSION GROWTH, AND ORGANOGENESIS OF *ACACIA KOA* TISSUE

Basal[a] medium	Solid[b] or liquid	Growth regulators[c]													Effectiveness[d]		
		Adn	IAA	IBA	C	NAA	2,4-D	B	Ben	D	K	BT	Z	GA	CM	Induction	Growth	Organogenesis
		mg/l													%			
SH	L						0.1	0.2			0.1					-	1	0
SH	L						0.2	0.1								-	1	0
SH	L						0.1	0.2								-	1	0
VW	L															0	0	0
VW	S							1								0	0	0
VW	S							5								0	0	0
VW	S										5					0	0	0
VW	S												5			0	0	0
VW			0.5												10	-	1	0
VW	L														10	0	0	0
VW	L							10							10	-	0	0
NN	S															0	0	0
K	L															-	0	1[e]
K	SL	20														-	0	1[e]
K	L										5					-	0	0
K	L	20													20	0	0	0
BL	S	20														-	0	0
LS	S	20				2.5									10	-	0	0
LS	S	20					0.2								10	-	0	0
W	S															0	0	0
W	S														10	0	0	0
W	S					2.5									10	0	0	0
W	S					2.5									10	0	0	0

[a] M: Murashige and Skoog 1962, M-: Murashige and Skoog without NH_4NO_3,
SH: Schenk and Hildebrandt 1972, VW: Vacin and Went 1949, NN: Nitch and Nitch 1969,
BL: Brown and Lawrence 1968, LS: Linsmaier and Skoog 1965, W: White 1963.

[b] S: solid, L liquid

[c] Abbreviations are on page xiv.

[d] Dash: No data, 0 - no change, 1 - occasional or poor, 2 - average, 3 - good.

[e] Organogenesis of roots only.

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