# TISSUE CULTURE OF PAPAYA, *CARICA PAPAYA* VAR. SOLO

by

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## APPROVAL BY THESIS DIRECTOR

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## ABSTRACT

Papaya, Carica papaya var. Solo No. 8, 5 mm long shoot tips and 3 mm long stem segments with their axillary buds were excised and cultured aseptically on modified Murashige and Skoog's medium. 1-10 ppm kinetin was added alone and in combination with 10-20 ppm IAA, 5-30 ppm IBA, and 1-5 ppm NAA to determine the proper treatment with which to obtain plantlets. Kinetin had a specific effect in inducing shoot formation, especially at a level of 1 ppm. Of the three auxins tested, IBA was superior in giving maximum growth of callus and in inducing root formation. The majority of induced roots were negatively geotropic and formed root hairs. Others which grew into the medium were devoid of root hairs. Cultured stem segments produced poor growth and leaf production. The largest number of complete plantlets were obtained after transferring shoots which had formed on the medium supplemented with 1 ppm kinetin to the medium containing 1 ppm kinetin plus 5 ppm IBA.

# INTRODUCTION

Papaya (Carica Papaya L.), which originated in tropical parts of the Old World, has long been used either as a source of food or for medicinal purposes. Besides its popularity as a fresh fruit, it is used for making canned food, jam, crystalized fruit, pickles, ice cream flavoring, and carbonated soft drinks (Ochse et al., 1961). Papain, a proteolytic enzyme, extracted by scarifying the skin of unripe fruit, is used by the medical profession wherever a digestive aid is required. It is used for chronic diarrhea in children, duodenal ulcers, a cure for ringworm, for chronic eczema, in face creams, in treating pyorrhea with its digestive action on the unhealthy pus of teeth, on tumors in cases of cancerous growths, and to bring on abortion if used in large doses (French, 1972). Papain as a meat tenderizer has long been used either by applying it directly on the meat or by injecting it into the blood stream of beef cattle before slaughtering. It is also reported that papain can be used as a blood anticoagulant (Pillai, 1955) which gives it the potential to be used in digesting blood stains on wool clothing because it revives the wool fiber rather than

breaking it. Apart from the use of papaya leaves for tenderizing meat by wrapping the meat in the leaves and leaving it overnight, leaves also can be used for making tea after they are fermented. Lloyd (1972) reported that papaya fruit and seeds have a high level of nitrate reductase enzymes. Hine, Aragaki, and Tokuraga (1965) took advantage of the proteolytic activity of papain by using it to stop the mobility and causing the death of zoospores of Phythium parasitica, Phythium aphanidermatum, Phytophthera parasitica, and Allomyces arbuscula. It has been reported by Badami and Daulatabad (1967) that papaya seeds are a good source of oil consisting of lauric, myristic, palmitic, stearic, archidis, behenic, hexadecenoic, oleic, and linoleic acids. El-Tayab, Marquis, and Kucerova (1974) have reported the richness of papaya seeds as a source of aglycone of glucotropaiolin benzyl isothiocynate (BITC) for its antibiotic and pharmacological activities and its toxicity. The useful properties of papaya are not limited to fruit, seeds, and leaves, but also are found in male and female flowers in the form of thirteen major amino acids (Kasinathan, Ramakrishman, and Srinivsan, 1965).

All of the above points establish that papaya as a very important crop. At the present time, however, the only satisfactory way of propagating this plant is sexually by

seeds. Tissue culture has the potential of being a satisfactory asexual method for the reproduction of papaya plants. Some investigators, however, have succeeded in propagating this species either by cuttings (Allan, 1964; Sen Gupta and Chattopadhaya, 1954; Traub and Marshall, 1937), grafting (Lang, 1969; Riccelli, 1963; Jimenez, 1959; Hancock, 1940), or by embryo culture (Phadnis, Budrukkar and Kaulgud, 1970). Grafting has not been successful since the hollow papaya stem makes it susceptible to breakage under windy conditions. Due to the nonbranching growth habit of the plant, on the other hand, the amount of stem cutting materials is limited. Papaya callus has been induced by transferring and subsequent subculturing of 1½ inch seedlings for periods of two years using modified White and Risser's medium containing 0.4 ppm of 2,4-dichlorophenoxyacetic acid (Medora, Campbell, and Mell, 1973). The intent of Medora and his colleagues was not to propagate the plant but only to prove the presence of proteolytic activity in the papaya callus.

Callus tissue is nothing more than a mass of undifferentiated paranchyma cells. Cambium tissue, if formed in this callus under suitable conditions, will differentiate into xylem and phloem. Such differentiation can be induced by grafting a bud where callus differentiates in the grafting

union. This also can be done in the absence of a bud merely by supplying the tissue with appropriate concentrations of growth regulators and nutritional requirements. Skoog and Tsui (1948) found that adenine induces bud formation. Naphthaleneacetic acid (NAA) stimulates root formation. The suggestion of Skoog and Tsui was that almost complete control of growth can be obtained by the application of different concentrations of auxin and cytokinins to the synthetic media.

Utilizing the above information it should be possible to propagate papaya plants by means of tissue culture.

The purpose of this study was an attempt to propagate papaya plants by culturing the shoot tips and the axillary buds. The effect of growth regulators in inducing shoots plus roots and/or callus from the cultured tissues was studied. If complete plantlets could be obtained by this technique, then it would be possible to: (1) eliminate the problem of genetic variability in plants obtained from seeds while maintaining desirable characteristics; (2) produce pathogen-free papaya clones; (3) accelerate the slow rate of reproductivity of this species; (4) obtain the desired quantity of female plants. Except in the case of bisexuals, 50% male and 50% female plants are usually obtained by sexual propagation. The desired ratio in a

papaya orchard is one male to every ten to fifteen females. Propagation by meristem culture, as a vegetative process, eliminates many of the problems encountered with sexual propagation.

# LITERATURE REVIEW

## General

Culture *in vitro* of plant cells, tissues, and organs has stimulated the interest of botanists for many years. The history of plant tissue culture goes as far back as 1902 when the German botanist, Haberlandt, first attempted to grow plant cells on a synthetic medium. His failure can be attributed principally to two factors: the first being the use of materials we now know are difficult to culture; the second being that the use of nutritional requirements and growth regulators were not clearly understood. However, Haberlandt was the first in formulating the concept of totipotency of plant cells. Many investigators have tried to cultivate plants by means of this technique. Tulecke and Nickell (1959) were the first to successfully grow a number of plant tissues by submerged culture.

Plant tissue culture can be catagorized into: plant culture consisting of culturing seedlings or larger plants; embryo culture using isolated mature or immature embryos; organ culture including isolated organs such as roots, stems, leaves, flowers and flower parts, fruits, and bark; tissue

or callus culture using tissues arising by proliferation from explants of plant organs; and finally suspension culture of isolated cell or cells suspended in a liquid medium.

## Embryo Culture

Embryo culture can be considered as the oldest method used. Many investigators have tried excising embryos of different species for the purpose of studying their structure and physiology. The first attempt to culture excised embryos on a nutrient solution was made by Brown and Morris (1890). Orchid seeds, which, due to their lack of stored food are little more than naked embryos, were germinated successfully on nutrient agar by Knudson (1922). The main objective of embryo culture is to make use of embryos which would otherwise abort if left to develop within the fruit. Laibach (1929) demonstrated, for the first time, the possibility of using this technique in genetic studies. In certain interspecific crosses in Linum, the fruit was found to be shrunken and the seed inviable, but when the embryos of such seeds were excised and placed on a sucrose medium, germination was induced. Overcoming seed dormancy is the other advantage of embryo culture. Seeds of some horticultural crops may remain dormant for several years. This was observed by Randolph (1945) who utilized embryo culture to obtain

seedlings of iris whose seeds otherwise remain dormant for several years. Another significant contribution was made by Graifenberg (1973) who cultured excised embryos of Rosa canina on modified Knudson's medium to overcome its long dormancy and its hard seed coat. Embryo culture also offered opportunities for some workers to shorten the breeding cycle of some deciduous trees (Lammarts, 1942), studying after ripening period of peach seeds (Kester, 1953), and vernalization in rye seeds (Gregory and DeRopp, 1938). Phadnis, Budrukkar, and Kaulgud (1970) successfully cultured mature papaya embryos in White's medium containing 0.05 ppm Kinetin with or without 0.1 ppm each of geberellin ($GA_3$) and indole-3-acetic acid (IAA).

## Excised Root Culture

Excised root culture has been mainly used to study the physiological processes in roots such as measuring their growth rate, respiration, protein synthesis and solute uptake. Street (1957) and Mitra (1968), however, were successful in propagating tomatoes and Rauvolfia serpentina by using this technique. In culturing roots of different species, the most commonly used nutrient media are modifications of White (1943).

## Culture of Flowers and Flower Parts

Ovules and ovaries of some species have been cultured on different synthetic medium to produce either viable seeds or plantlets. Johri (1961) cultured ovaries, ovules, and embryos of several angiosperms including onion, candytuft, hollyhock, zephyranthes, opium poppy, and *Citrus microcarpa*. Small grape plants were produced by Radler (1964) from culturing ovary tissue on a basal medium. Button and Borman (1971) induced Washington Navel orange pseudo bulbils and subsequent differentiation of embryoids by cutting unpollinated and unfertilized ovules on a basal medium supplemented with adenine and malt extract to produce plantlets after transferring these embryoids to another basal medium containing gibberellic acid. Niimi (1973) produced five to six viable seeds from each cultured ovule of *Petunia hybrida* on a modified Nitsch basal medium.

Culturing flowers and flower buds has contributed to the advancement of plant physiology and genetics. Conversion of cucumber male flowers into female ones has been achieved by Galun, Jung, and Lang (1962) by culturing excised potentially male floral buds on modified White's medium with various levels of IAA and $GA_3$. Viable onion seeds

were successfully developed by culturing excised flowers two days after pollination (Guha, 1962).

Ram and Wadhi (1966) accomplished the reversion of excised Kalanchoe pinnata pers. floral buds into vegetative state by culturing on modified White's medium supplemented with 2,4-D and coconut milk; roots and shoots were initiated on the resulting vegetative buds. Pow (1969) devised an excellent method for making sterile tissue cultures from small portions of cauliflower curd, hardening and potting the resulting plantlets which attained maturity and formed curds. This process eliminated the risk of losing valuable plants which will not survive in the field or under glass for breeding at the curding stage. Producing complete flowers besides the restoration of pollen viability from completely male-sterile species was done by Majumdar (1970) from culturing young Haworthia and Astroloba floral buds on White's basal medium supplemented with IAA, kinetin, and coconut milk. Producing completely developed fruits from culturing pollinated flowers was achieved by using sterile culture technique on media combined with growth regulators (Bajaj and Collins, 1967).

## Mutations Perpetuated by Tissue Culture

Tissue culture, as a potential vegetative method of propagation, may provide mutants. Mutations so produced might be preferable in producing new clones with good merits. Shigmatsu and Matsubara (1972) induced chimeric mutants by subjecting to gamma rays small pieces of mature leaves of Begonia rex cultured on White's medium. Two of the 30 plants so produced showed chimeric mutation leaves which in turn produced intact plants of green leaves with silver spots as compared with the silver-white leaves of the original ones.

## Meristem Culture

Meristem culture has been used since 1922 when Robbins cultured stem tips of peas, corn, and cotton in Pfeffer solution. One advantage of this method is that meristem tips may be free of diseases (Hartman and Kester, 1968) which may provide the opportunity to produce pathogen-free plants. The oldest successful meristem culture experiment was conducted on orchid plants (Morel, 1960).

Many other ornamental plants have been produced since then such as carnation, chrysanthemum, buddleia, dahlia, hydrangea, pelargonium, phlox, saintpaulia, freesia, gladiolus, lily, iris, narcissus, tulip (Paludan, 1971),

diffenbachia (Zimmer, Koch, and Phalow, 1973), geranium Boettcher and Hildebrandt, 1964), caladium (Hartman, 1974), and glaxinia (Haramaki, 1971). Hyacinth, on the other hand, was propagated by culturing small pieces of leaf, or small stem, taken from sprouted bulbs which in turn yielded bulbuls with roots (Hussey, 1974). Hussey's method of propagating hyacinth could be adapted for other kinds of bulbous plants.

Meristem culture has been used for propagating some of the vegetable plants such as pea (Kartha, Gamborg, and Constabel, 1974), tomato (Hussey, 1971), chinese cabbage (Lee, Chung, and Han, 1973), rhubarb and horse-radish (Paludan, 1971).

Meristem culture has also been applied successfully to some trees, vines, and grasses such as apple (Walkey, 1972), some prunus species (Quoirin, Boxus, and Gaspar, 1974), strawberry (Papov, 1974), hop (Vine and Jones, 1969), raspberry (Putz, 1971), and sugar cane (Roth, 1969). Attempts to culture apical meristems of date palm have not been successful. Shoots were initiated for this species but without roots (Reuveni, Adato, and Linien-Kipnis, 1972).

Producing new strains of plants by the use of meristem culture technique is one of the valuable sidelights which has been seized upon by some investigators. An

excellent example of that is the work of Maia and his colleagues (1973) who succeeded in producing two strains of Abrial lavandin resistant to dieback disease. McCown, Beck, and Struckmeyer (1967), using sterile culture, micrografted vegetative stem tips of Chrysanthemum morifolium into the placental tissue of pepper fruits. A non-vascular union between the tissues was produced within 20 days and subsequent growth of the stem tip was observed as cellular expansion, division, and primordia formation. Their technique may prove applicable for in vivo studies of the mechanism of graft acceptance in plants as well as for biochemical studies of the interaction between adjacent tissues.

## Callus Culture

It has recently been possible to produce induced differentiation of callus, derived from different plant explants, into roots, shoots, and finally into plants. Differentiation has been demonstrated in callus of asparagus (Wilmar and Hellendoorn, 1968), geranium (El-Nill and Hildebrandt, 1971), coffee (Staritsky, 1970), citrus (Chaturvedi and Mitra, 1974), Mesembryanthemum floribundum (Mehra and Mehra, 1972), endive and parsley (Vasil, Hildebrandt, and Riker, 1964), lilium (Sharp and Raskin, 1971), Pelargonium (Chen and Glaston, 1967), Rauvolfia serpentina (Mitra and Chaturvedi, 1970),

tobacco (Skoog and Tsui, 1948), Cheiranthus cheiri (Khanna and Staba, 1970), pumpkin (Jelaska, 1974), carrot (Steward, Mapes, and Mears, 1958), gladiolus (Ziv, Halevy, and Shilo, 1970), freesia (Bajaj and Pierik, 1974), eggplant (Raina and Iyer, 1973), potato (Svobodova, 1966), Solanum dulcamara (Zenkteler, 1973), chrysanthemum (Hill, 1968), convulvulus (Earle and Torrey, 1965), Anthurium andraeanum (Pierik, Steegmans, and Van Der Meys, 1974), and almond (Mehra and Mehra, 1974).

Callus culture has also made it possible to conduct experiments in vitro which are difficult to achieve in vivo. For example, excellent results were obtained by Scaramuzzi (1969) who grafted strips consisting of phloem plus cambium of pear on quince. Callus was formed at the grafting union which, unlike those achieved in vivo, were good to perfect and consist of equal contributions from the two species. Similar work was also done on grapevine, peach, persimmon, pear, and apple (Fujii and Nito, 1972). The callus fusion of those plants (nil to complete) depended on the taxonomic closeness of the two partners with one exception which was the close union between chestnut and persimmon. Fujii and Nito (1972) agree, however, on the point that callus fusion, although important, is not the only factor in graft formation.

Research has been done in producing edible plant tissues (callus) by culturing different explants on a synthetic medium (Hildebrandt et al., 1963). Although it is not feasible at this time, it provides a potential for producing food stuffs on a massive scale should it be required by future need. Other experiments revealed the production or biosynthesis of beneficial plant constituents by means of tissue culture. Among those compounds are alkaloids (Babcock and Carew, 1962), amino acids (Seitz and Hochster, 1964), antibiotics (Mathes, 1963), carbohydrates and organic acid (Mohran Ram and Steward, 1964), enzymes (Straus and Campbell, 1963), flavonoids (Kordan and Morganstern, 1962), glycosides (Sargent and Skoog, 1961), growth regulators (Nickell, 1958), terpenoids (Arreguin and Bonner, 1950), tannins (Ball, 1950), lignins (Dougall, 1962), and pigments (Hildebrandt et al., 1963).

## Media

The importance of nutrient laden media is that the excised plant parts soon deplete the nourishment in their own tissues unless supplied with a new source. A wide variety of media have been used for the purpose of culturing different types of tissues and organs. The types of media differ according to procedure being used, plant species,

plant part, and the developmental stage of the excised plant part. However, components of most nutrient media being used so far can be classified into: organic (amino acids, sugars, vitamins, growth regulators), inorganic macro-elements (P, Ca, K, Mg, S, N), inorganic micro-elements (B, Cu, Mn, I, Fe, Zn), unidentified mixtures of substances such as coconut milk and yeast and plant extracts, and sometimes coagulating agents (mostly agar).

Among the most widely used basal media are White's, Murashige and Skoog's, Knudson's, Wolter and Skoog's, Morel's, and Berthelot and Knop's solutions. Others which are not so widely used include Morel and Martin's, Miller's, Nitch's, Novel's, Vacin and Went's, Smirnov's, Linsmaier and Skoog's, Quak's, Baker's, Pfeffer's, Gartheret's, and Ball's.

Many modifications of the basal media have been made by either changing the concentration of a specific compound or by adding new ones in order to supply the excised parts of a specific species with required nutrients. Paludan (1971), however, tried to classify certain media for specific purposes. He found that nutrient media based on Berthelot's and Knop's solutions were satisfactory for growing herbaceous ornamentals; callus and tissue culture of herbaceous and woody ornamentals, bulbs, corms, and vegetables, on the

other hand, are best grown on Murashige and Skoog's nutrient medium. Modified Gartheret's solution has long been used for the production of carnations from excised shoot tips.

As far as the physical properties are concerned, there are three kinds of media: liquid, semi-solid, and solid. Using liquid media usually requires a shaking mechanism in order to supply adequate aeration for the cultured tissues. Another technique associated with the use of liquid media consists of culturing the tissue on an ashless filter paper wick which serves as a passive support and is usually used with carnations' shoot tips for better root formation (Paludan, 1971). A good example showing the effect of the physical properties of the media on the course of differentiation of the excised parts was shown by experiments conducted by Vasil and Riker (1964) and Vasil and Hildebrandt (1966) on endive: (a) in liquid media, small masses of tissue or embryoids formed from single cells which then differentiate roots and shoots; (b) in semi-solid media, roots and shoots formed from localized meristematic area. The course of development of embryoids or buds obtained in tissue grown on agar media, however, was not well understood by those investigators.

## Growth Regulators

In the past, tissue culture relating to the culture of human and animal tissue was more advanced than that of plants. At the present, however, due to the discovery of a number of plant growth regulators the plant tissue culture specialists are rapidly catching up. There are three major classes of plant growth regulators which have been widely investigated: auxins, cytokinins, and gibberellins. Many physiologists have preferred the procedure of isolating tissue *in vitro* for such investigations. This rare technique permits the separation of cells from the complex interreaction to which they are subjected in the intact plants.

Auxins, as any plant growth regulator, are normally added at low concentration to plant culture media in order to obtain their appropriate effects. Most commonly used for this purpose are the natural auxin indole-3-acetic acid (IAA) and the synthetic ones naphthalene-1-acetic acid (NAA), indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D).

Auxins have been associated with plant cell wall expansion and cell elongation (Tanimoto and Masuda, 1971),

cell division (Clutter, 1960), increased RNA synthesis and decreased RNase activity (Gwazdz, 1973) and increased mitochondrial activity (Sarkissian and McDaniel, 1966).

Cytokinins are defined as compounds that induce cell division in plant cells (Wilkins, 1969). There are either naturally occurring cytokinins: zeatin and 6-methylaminopurine (MAP) or synthetic: 6-benzyladenine (BA), 6-benzylaminopurine (BAP), and 6-(3-methyl-2-butenylamino) purine (2ip), and 6-furfurylaminopurine (kinetin) which was obtained from DNA as a starting material.

Skoog and Tsui (1948) showed that auxins IAA or NAA induce callus growth and root initiation but inhibit bud formation. They also showed that adenine does the opposite i.e., induces bud formation and inhibits root initiation. Their conclusion was that nearly complete control on the formation of roots, buds or undifferentiated growth of tissues can be obtained by the application of different proportions of auxin and adenine to their medium. The same conclusion was reached by Bajaj and Pierik (1974) and Fridberg (1971). Pillai and Hildebrandt gave a good description of the anatomical changes accompanying the differentiation of geranium callus on a medium supplemented with kinetin and NAA in their paper which appeared in 1969a.

Zwar and Brown (1968) did an elaborate experiment on locating where a particular cytokinin or auxin was being incorporated. They found that artichoke tuber tissue grown with labelled kinetin showed pronounced labelling of both nuclei and cytoplasm, whereas those grown with labelled 2,4-D showed dense labelling of nucleoli and comparatively little labelling elsewhere. Zwar and Brown's experiment may shed new light in explaining precisely how plant growth regulators show their physiological mode of action.

Selecting a particular type of auxin or cytokinin depends on the result of its physiological action, stability, persistance and mobility. Some researchers have preferred the use of synthetic auxin, IBA, or NAA over the natural occurring one, IAA, due to their greater chemical stability and low mobility. On the subject of auxin mobility Kulescha (1972) working with Jerusalem artichoke showed that IAA, NAA, and 2-4-D were incorporated in the cultured tissue after 1, 2-3, and 48 hours respectively. Greater chemical stability of any growth regulator provides improved persistance and extended action. However, the type of tissue, the endogenous level of natural occurring hormones, and the complex interaction of those hormones define the particular type and concentration of growth regulator to be used. Papov and

Butenko (1970) concluded that the use of kinetin plus NAA supported callus of apple and sour cherry better than adenine plus 2,4-D. Winton (1970) preferred to use 6-benzylaminopurine (BAP) plus 2,4-D to produce Populul termuloides trees from callus tissue. Pennazio (1973) confirmed the conclusion that explants of some species contain sufficient endogenous levels of growth substances to support their growth into complete plants by successfully producing carnations by means of shoot tip culture without the addition of any of the growth regulators to his media.

Some workers (Boettcher and Hildebrandt, 1964, for example) found that the addition of some plant extracts and natural substances to synthetic media would give better growth and differentiation of explants cultured in vitro. Coconut milk (liquid endosperm) is the most widespread natural substance used in tissue culture. Salisbury and Ross (1969) mentioned that the natural fraction of coconut milk has no activity but it is essential for cell division. Salisbury and Ross' observation support the conclusion that coconut milk, and some other plant extracts, contain some substances having a regulatory effect on the cultured tissue besides the familiar nutritional materials. A good

report on the composition of the coconut milk can be found in the paper of Tulecke and his co-workers (1961).

## MATERIALS AND METHODS

### Seed Germination

Papaya seeds (*Carica papaya* var. Solo No. 8, supplied through the courtesy of Dr. Hamilton of the University of Hawaii) were germinated in flats in a greenhouse. The flats were kept in shade and under misting. Temperature ranged from approximately 79°F during the day to 70°F during the night. The resulting seedlings were moved to an area where they would receive sunlight and were permitted to remain in the greenhouse until they reached 6 cm in height. Fish emulsion fertilizer (5-2-2) was applied on two occasions in an amount of ½ teaspoonful per quart of water.

### Tissue Excision and Sterilization

Shoot tips 5 mm long weighing 3 ± 0.5 mg and stem segments (with their axillary buds) 3 mm long weighing 6 ± 0.5 mg were excised by using a sterilized razor blade. Excision was accomplished under a surface-sterilized low power binocular dissecting microscope after the leaves were stripped off the plants.

The excised tissues were surface sterilized by soaking them in household bleach, diluted with distilled water

in a ratio of 1:9, for 15 minutes then rinsed in sterile distilled water.

## Media Selection

The first medium tested was the one used by Ball (1946). The use of this medium ceased when it was discovered that it did not promote growth of the papaya tissue.

Other media were considered; and Murashige and Skoog's (1962) was selected. Modifications of this medium included the use of casein acid hydrolysate, kinetin, and the auxins IAA, IBA, and NAA with the latter four in different concentrations. The addition of the chemical polyvinylpyrrolidone to this medium was attempted but was abandoned due to its lack of response. Difco agar in a concentration of 9.2 g/l was added to the culture vessels before dispensing the medium to them. Demineralized water was used throughout the experiment for media preparation. Media were either used on the same day or kept refrigerated at circa 40°F for later use.

## Media Sterilization and pH Adjustment

Media sterilization was accomplished by autoclaving 15 minutes under 17 pound pressure at 255°F. The pH of

nutrient media was adjusted to 5.5 ± 0.2 with NaOH or HCl prior to autoclaving. Occasional check tests revealed that autoclaving had no significant effect on pH of the medium.

## Tissue Transfer to Culture Vessels

Pyrex culture tubes (2.5 x 15 cm) were used as culture vessels, and each tube contained 25 ml of the medium. Only tissues which achieved good growth were transferred to 125 Erlenmyer flasks containing 75 ml of medium. Stoppers of plastic foam were used rather than conventional cotton plugs.

One shoot tip or stem segment was used per culture and 10 replicants (5 tips and 5 stem segments) comprised each treatment. The tissues were transferred to the culture tubes, with the nutrient medium, in an inoculation chamber sterilized by washing with Septicol and alcohol. Position of the tissues was checked by head gear optical glass binocular magnifier and any necessary reorientations were made with a sterilized round tipped needle.

## Tissue Incubation

The cultures were kept in a growth chamber at 85 ± 2°F under cool white fluorescent tubes giving 460 foot-candles at the level of the tissues in the tubes. The lights were turned on at 8:00 a.m. and off at 4:00 p.m. i.e., on a

basis of 8/16 light-dark cycle. No study was made on the importance of light or temperature. It is apparent, however, from occasional variation in the above conditions that considerable latitude was possible in light and temperature without seriously altering either growth or differentiation of the culture. Nevertheless, the culture tubes were occasionally rotated to eliminate any bias due to temperature and light differences in the growth chamber.

## Growth Regulator Studies

Kinetin, 6-furfurylaminopurine[1], was added to the basal medium in a range of 1 mg/l to 10 mg/l combined with each of the following auxins: indole-3-acetic acid[2], (used in a range of 10 mg/l to 20 mg/l), 3-indolebutyric acid[3], (used in a range of 5 mg/l to 30 mg/l), and naphthalene acetic acid[4], (used in a range of 1 mg/l to 5 mg/l).

Growth measurements for each cultured tissue consisted of the number of leaves and the ratios of the final to initial weights; and these ratios were given the notations,

1. Obtained from Calbiochem
2. Obtained from Sigma Chemical Company
3. Obtained from Matheson Coleman & Bell
4. Obtained from Nutritional Biochemicals Corp.

growth values. All measurements were made after the tissues remained 4 weeks on the same culture media.

# RESULTS

## Effect of Amount of Agar Added

Because of the tendency of agar to partially precipitate in the bottom of the tubes after autoclaving, the amount added to them was 9.2 g/l as opposed to 6.4 g/l when 125 ml Erlenmeyer flasks were used. Smaller amounts of added agar produced a slurry and larger amounts caused a hard gel. Comparisons between cultures placed on agar and those floated on unaerated liquid media indicated the growth was better on the solid ones.

## Effect of Tissue Size and Sterilization

Shoot tips used in the experiment varied in size from 0.5 mm to 5 mm. Those smaller than 5 mm proved unsatisfactory because, during transfer, they were difficult to see and subject to injury. Also, the sterile distilled water which was used to keep them moist during transfer caused some of them to burst. The 5 mm shoot tips proved to be exempt from the above disadvantages, and therefore found to be the ideal size. Approximately 4% contamination occurred throughout the experiment.

## Kinetin and Indole-3-Acetic Acid Experiment

### Growth Value

The nature of the interaction between kinetin and IAA and their effects on the growth values of cultured shoot tips and stem segments was studied with the two substances supplied in different combinations and proportions. Results of the experiments are presented in Figures 1 and 2. Figure 1 shows that kinetin increased the growth values of the cultured shoot tips in the presence and absence of IAA and that optimum yields were obtained with 1 ppm concentrations regardless of IAA concentration. However, IAA increased the yields in combined treatments with high kinetin levels. The effect of kinetin was to induce the development of shoots without any callus formation. IAA, on the other hand, mainly induced callus production both in the presence and absence of kinetin. In general, the callus was watery, soft and friable. It can be seen from Figure 1 that maximum yield of callus in the case of IAA alone was at 15 ppm. Interaction between 1 ppm kinetin and 10 ppm IAA gave the highest growth value. It also can be seen that at the level of 1 ppm kinetin, increasing the concentration of IAA from 10 to 20 ppm decreased the growth value by almost four-fifths.

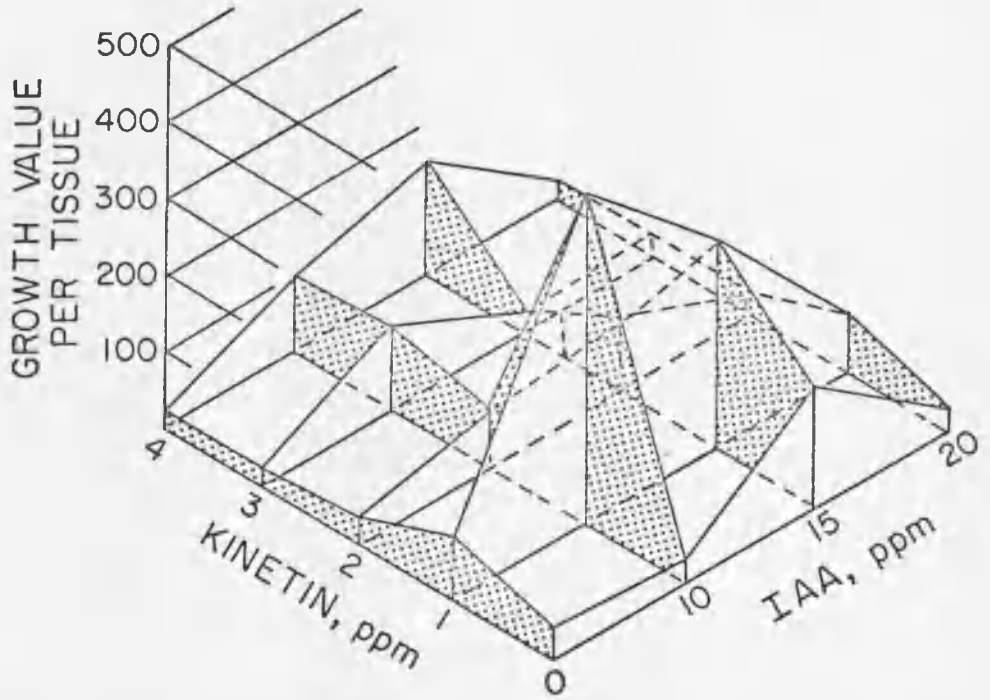


Figure 1. Growth of Culture Shoot Tips as Affected by Adding to the Medium 1-4 ppm Kinetin and 10-20 ppm IAA Alone and in Combination.

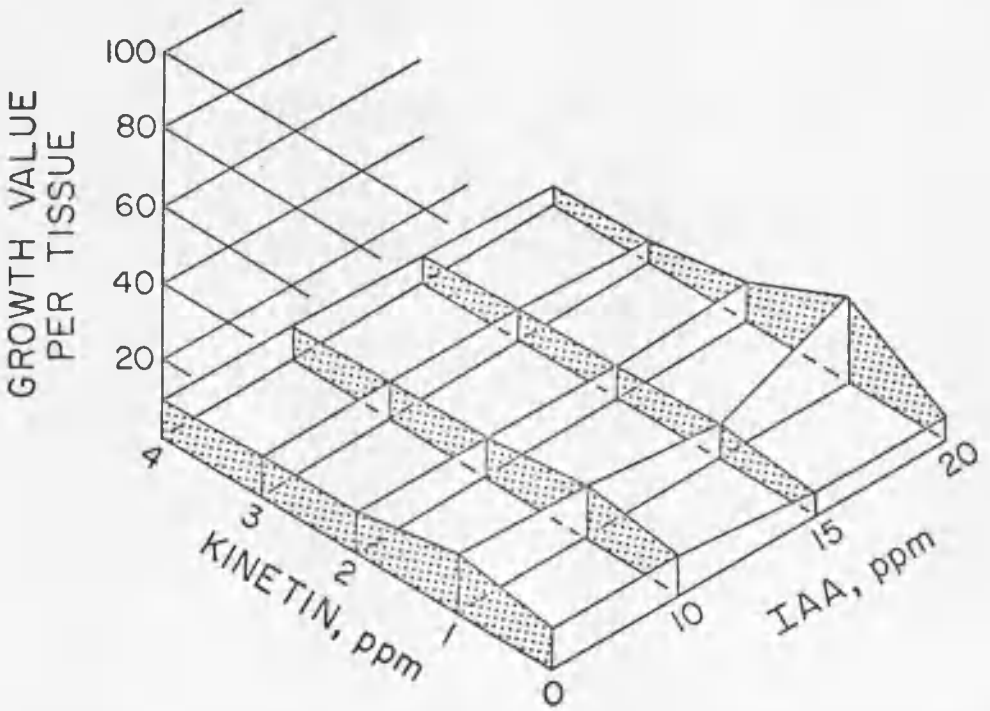


Figure 2. Growth of Cultured Stem Segments as Affected by Adding to the Medium 1-4 ppm Kinetin and 10-20 ppm IAA Alone and in Combination.

In experiment with stem segments (Fig. 2) the overall growth value of all treatments used was much less than that of shoot tips (Fig. 1). It should be noted, however, that in contrast to the growth of shoot tips, which was optimal at the combination of 1 ppm kinetin and 10 ppm IAA, the maximum yield in case of stem segments was at the point where 1 ppm kinetin interacted with 20 ppm IAA. It is worthy of being mentioned that no callus was obtained from cultured stem segments except the treatment which gave the highest growth value.

Development of Leaves

Results based on the number of leaves developed per cultured shoot tip or stem segment are summarized in Figures 3 and 4, respectively. Figure 3 shows that the highest number of leaves per tip produced by kinetin alone was at 1 ppm and had a retarding effect if used at higher concentrations. It is apparent that in the absence of exogenous kinetin, the number of leaves increased with the level of IAA to a maximum at 15 ppm and then fell at 20 ppm. By interacting both substances, at the level of 1 ppm kinetin and 10 ppm IAA, it was possible to obtain the highest number of leaves. In comparison to Figure 3, increasing the concentration of kinetin in the absence of exogenous IAA had the

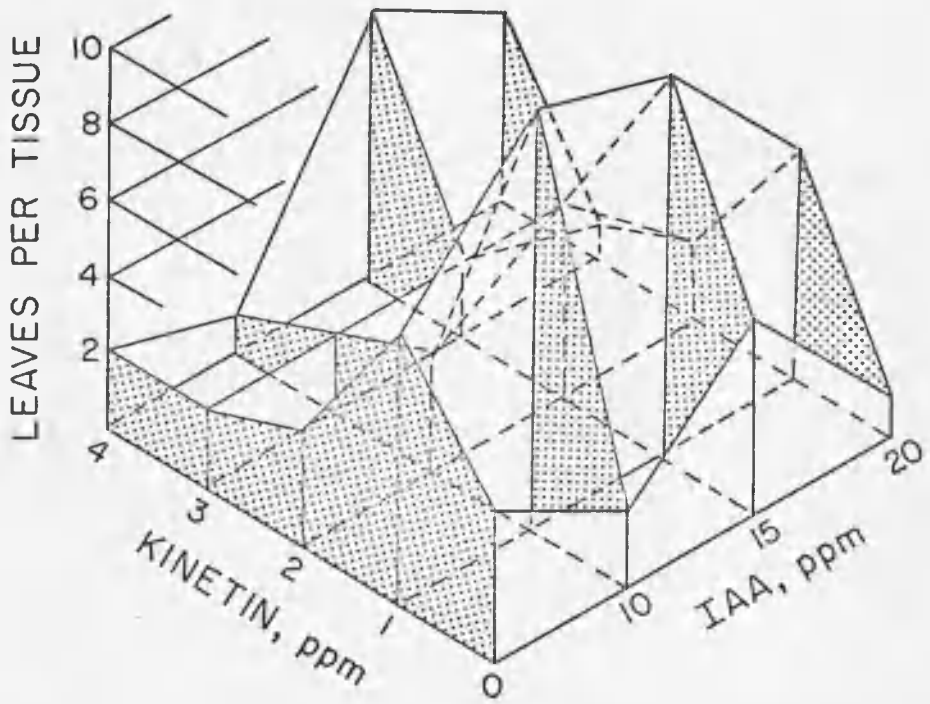


Figure 3. Number of Leaves Developed from Shoot Tips as Influenced by 1-4 ppm Kinetin and 10-20 ppm IAA Added to the Medium Alone and in Combination.

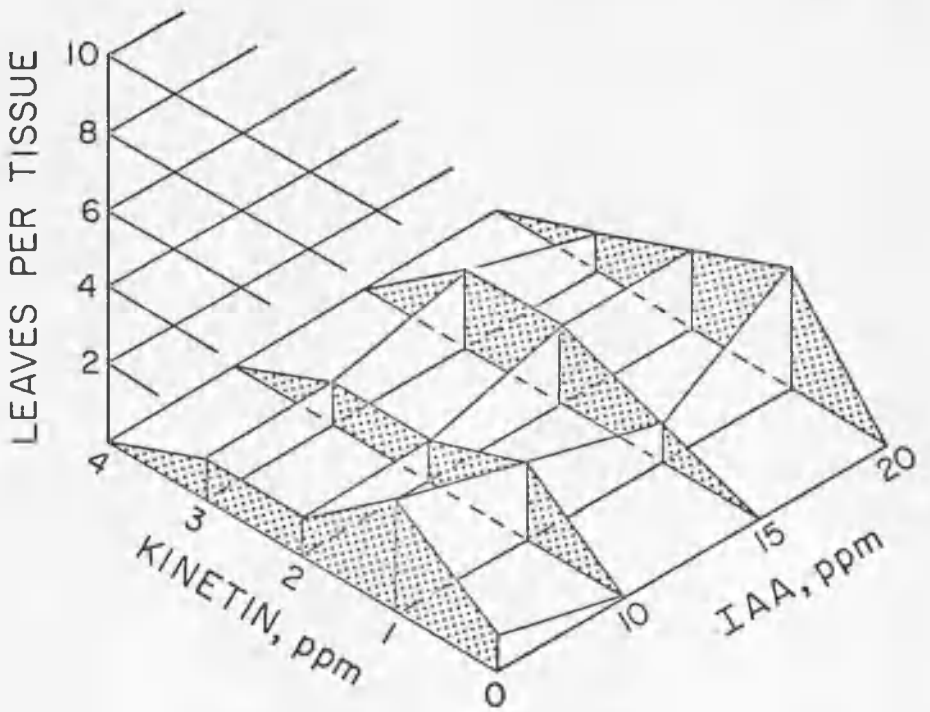


Figure 4. Number of Leaves Developed from Stem Segments as Influenced by 1-4 ppm Kinetin and 10-20 ppm IAA Added to the Medium Alone and in Combination.

same effect on the stem segments (Fig. 4) except that at the level of 4 ppm kinetin where it completely retarded any development of leaves. In general IAA, whether used alone or with 4 ppm kinetin, inhibited leaf development on the stem segments.

## Kinetin and 3-Indolebutyric Acid Experiment

### Growth Value

Effects of kinetin (from 1 to 4 ppm) in combination with 5 to 30 IBA on the growth of the cultured tissues are illustrated by Figures 5 and 6. It is clear from Figure 5 that increasing the concentration of IBA above 5 ppm brought about growth retardation in the absence of kinetin. In fact, the growth value of tissues cultured on the medium supplied with only 5 ppm of this auxin is 3.7 times more than that at 30 ppm. It also appears that kinetin resulted in higher yields and healthier tissue when added in combination with IBA than if added alone except in cases where kinetin is used in levels above 2 ppm. One main feature of this graph is to show that both 1 ppm kinetin and 5 ppm IBA were interacted to give the highest yield. Callus produced by using this auxin alone or in combination with kinetin was watery and friable with a few white glistening globes.

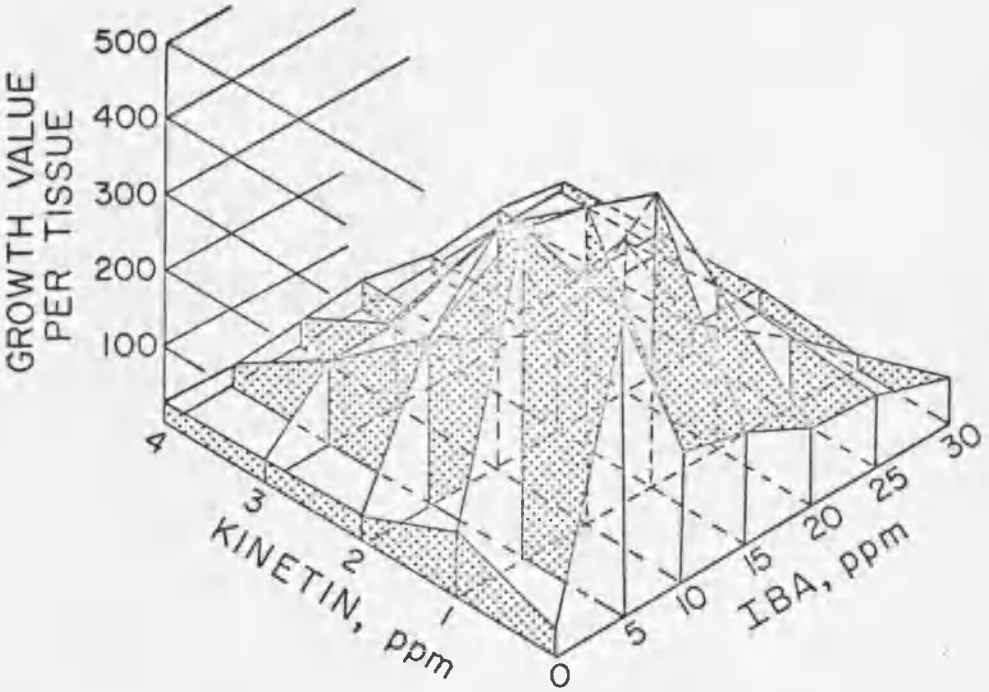


Figure 5. Growth of Cultured Shoot Tips as Affected by Adding to the Medium 1-4 ppm Kinetin and 5-30 ppm IBA Alone and in Combination.

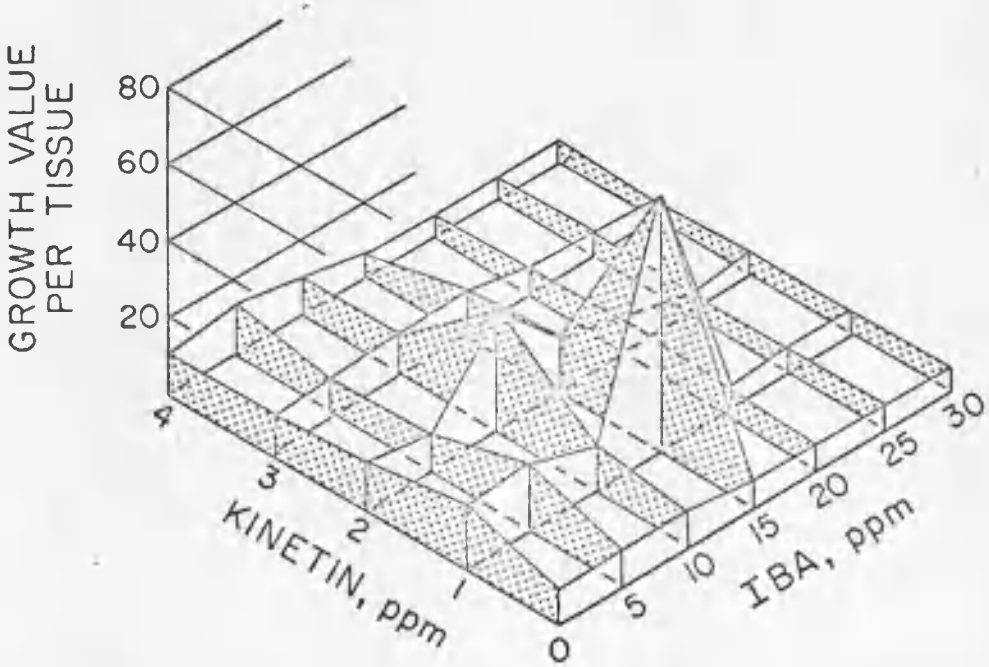


Figure 6. Growth of Cultured Stem Segments as Affected by Adding to the Medium 1-4 ppm Kinetin and 5-30 ppm IBA Alone and in Combination.

As with IAA, the overall growth value of treatments studied in their effect on stem segments (Fig. 6) was much less than that of cultured shoot tips (Fig. 5). Figure 6 shows that both kinetin and IBA did not increase yield of the tissue substantially if used alone. In fact, even the combination of both 1 to 4 ppm kinetin and 20 to 30 ppm IBA gave growth values not too different from that of the control without growth regulators added. Nevertheless, Figure 6 shows that with 1 ppm kinetin interaction with 15 ppm IBA gave the highest yield.

Development of Leaves

Figures 7 and 8, respectively, illustrate the effect of both kinetin and IBA, used alone and in combination, on the number of leaves developed per cultured shoot or stem segment. Figure 7 shows that kinetin gave the maximum number of leaves per cultured shoot tip when added alone, especially at 1 ppm, than in combination with IBA. Higher levels than 1 ppm kinetin were without benefit and were inhibitive. Figure 7 clearly shows that the control treatment resulted in higher numbers of leaves than most of those cultured on media where both kinetin and IBA were added together.

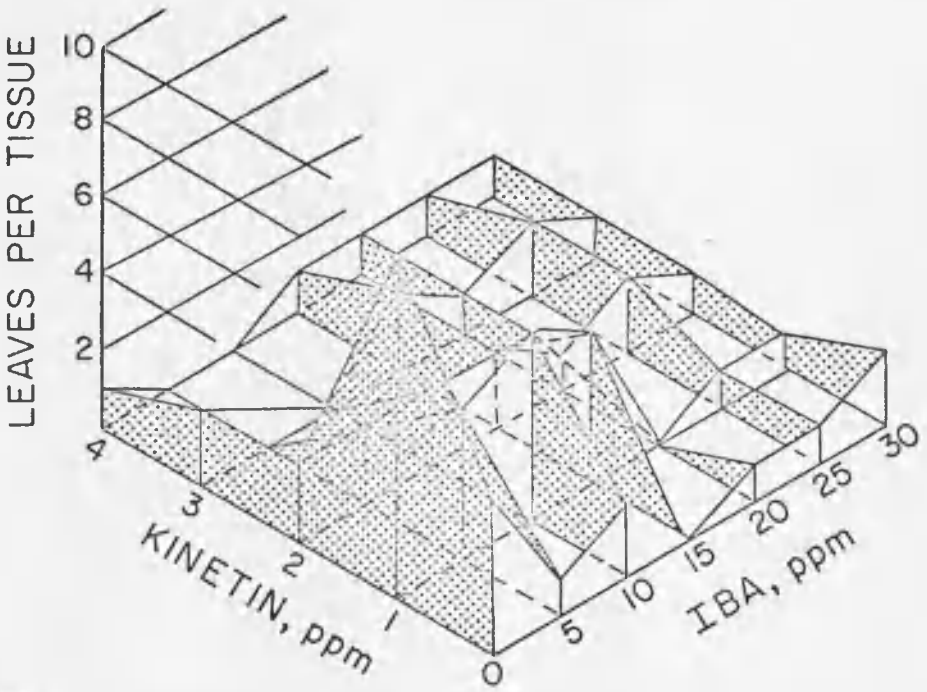


Figure 7. Number of Leaves Developed from Shoot Tips as Influenced by 1-4 ppm Kinetin and 5-30 ppm IBA Added to the Medium Alone and in Combination.

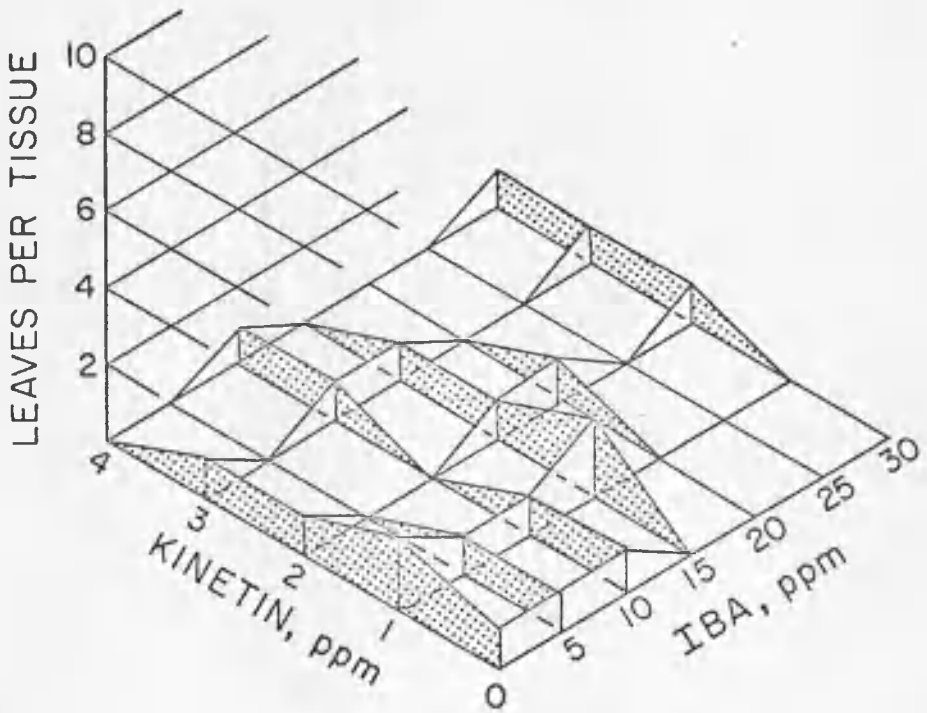


Figure 8. Number of Leaves Developed from Stem Segments as Influenced by 1-4 ppm Kinetin and 5-30 ppm IBA Added to the Medium Alone and in Combination.

In contrast to Figure 7, Figure 8 reveals that the number of leaves developing per cultured stem segment was much smaller than most of the treatments which were used in the case of the shoot tips.

Development of Plantlets

Shoots, devoid of callus, formed on both the control and the medium containing 1 ppm kinetin and were transferred after four weeks to a medium supplemented with 1 ppm kinetin plus 5 ppm IBA. Ninty per cent of those transferred shoots developed adventitious roots deep within the callus after 2-3 weeks from the date of the second transfer producing normal plantlets (Figs. 9 and 10). In most cases one plantlet was produced from each cultured shoot tip.

Some of the roots showed negative geotropism (Fig. 9), others grew down into the medium (Fig. 10). From Figure 9 it can be seen that roots growing above the medium were thin and produced secondary roots plus some root hairs. Others which grew into the medium were thick with secondary roots but were devoid of any root hairs (Fig. 10). It is not known about the exact origin of those roots. All that can be said is that they formed from regions deep within the callus near the base of the developing shoot.

Figure 9. Two Week Old Plantlet Developed After Transferring Shoot Which Had Formed on the Control to a Medium Supplemented with 1 ppm Kinetin Plus 5 ppm IBA.

Figure 10. Two Week Old Plantlet Developed After Transferring Shoot Which Had Formed on the Medium Supplemented with 1 ppm Kinetin to a Medium Containing 1 ppm Kinetin Plus 5 ppm IBA.


Figure 9. Plantlet from Control

Figure 10. Plantlet from Medium with Kinetin

It is of interest to mention that no callus was formed from those roots which grew above or into the medium. Callus was former, however, on some of the leaves of the growing shoots (Fig. 10).

## Kinetin and Naphathalene Acetic Acid Experiment

### Growth Value

Typical effects of serial combinations of kinetin and NAA on growth values of cultured tissues are summarized in Figures 11 and 12. 1 to 10 ppm kinetin was allowed to interact with 1 to 5 ppm NAA. Figure 11 shows that the peak yield obtained from treatment with kinetin alone was at 1 ppm. Adding kinetin to the medium at levels beyond 1 ppm has a repressing effect on the shoot tip's growth. All tested concentrations of NAA had a definite growth retardation which tended, however, to be counteracted by kinetin. Treatment with 1 ppm kinetin with 5 ppm of this auxin gave the highest yield among other kinetin-NAA combined treatments. Small amounts of white, friable, and sometimes firm callus was produced by interacting the two substances.

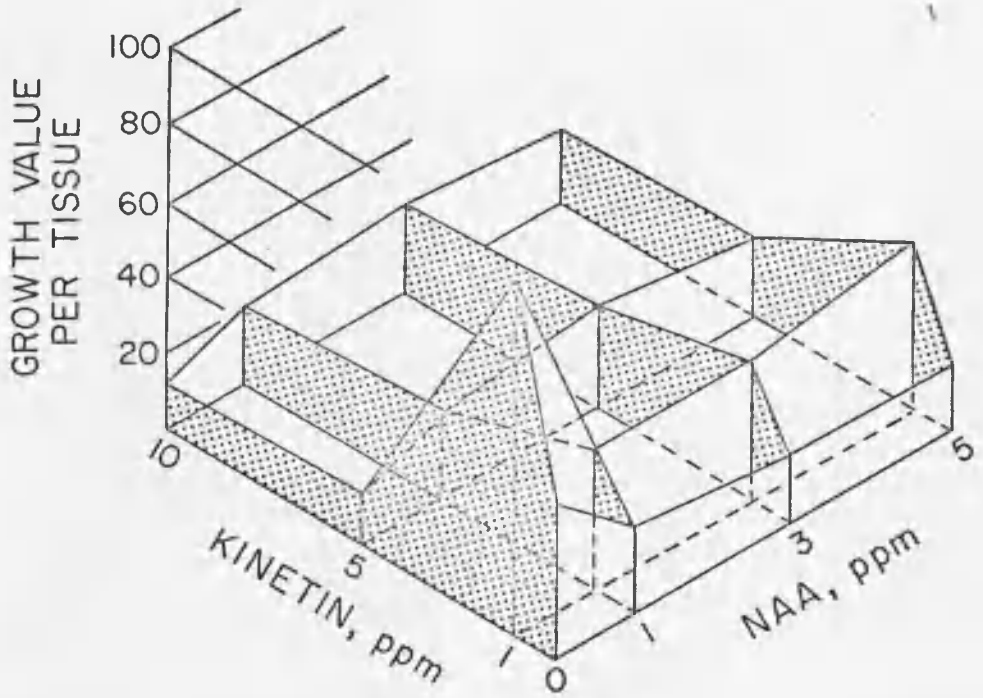


Figure 11. Growth of Cultured Shoot Tips as Affected by Adding to the Medium 1-10 ppm Kinetin and 1-5 ppm NAA Alone and in Combination.

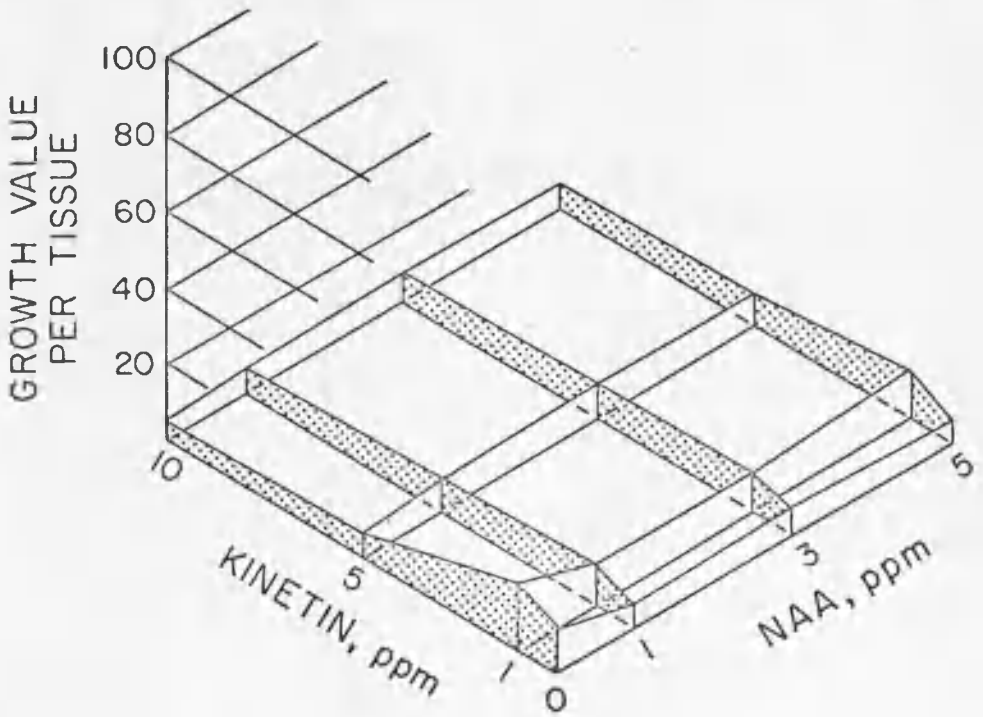


Figure 12. Growth of Cultured Stem Segments as Affected by Adding to the Medium 1-10 ppm Kinetin and 1-5 ppm NAA Alone and in Combination.

In contrast to Figure 11, Figure 12 shows that all tested treatments had no significant effect on increasing growth of cultured stem segments.

Development of Leaves

Kinetin added at a concentration of 1 ppm caused a significant increase in number of leaves developed from cultured shoot tips (Fig. 13). Yield of leaves fell to zero, however, when levels of kinetin was raised to 5 ppm or higher. All examined levels of NAA had a repressing effect which somehow was slightly counteracted by kinetin.

Except in the case of 1 ppm kinetin, kinetin and NAA whether used alone or in combination did not improve the leaf yield of the cultured stem segments (Fig. 14).

Development of Plantlets

Trials were made to transfer tissues treated with kinetin and NAA to both the control and the medium supplemented with 1 ppm kinetin plus 5 ppm IBA. Twenty per cent of the tissues treated previously with 1 ppm kinetin plus 1 ppm NAA and which transferred to the control were rooted. Figure 15 shows a sample of such tissues where both primary and plenty of secondary roots were formed but with very poor shoot production.

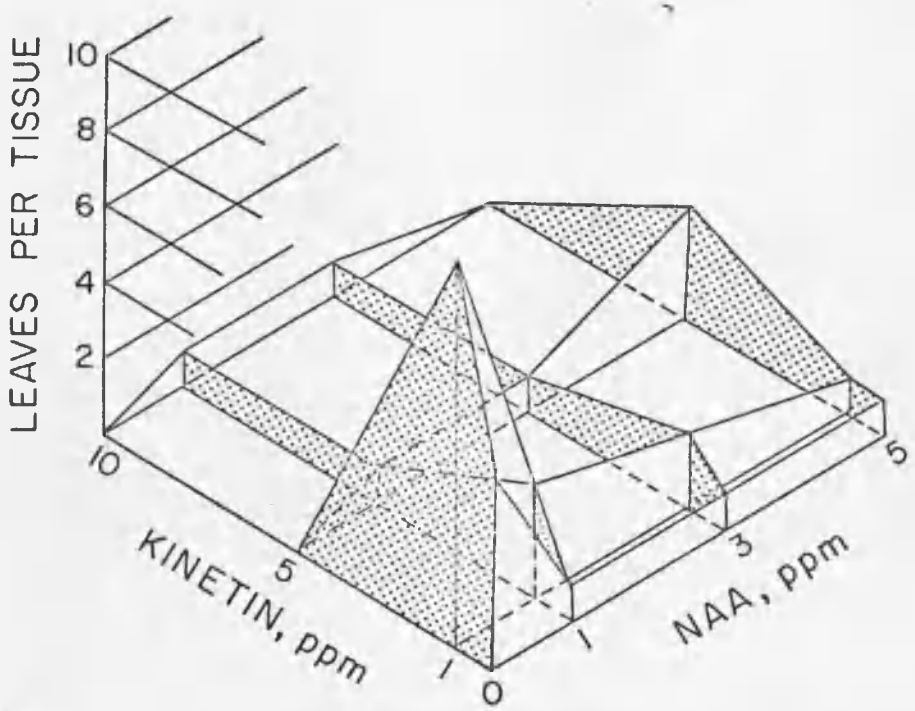


Figure 13. Number of Leaves Developed from Shoot Tips as Influenced by 1-10 ppm Kinetin and 1-5 ppm NAA Added to the Medium Alone and in Combination.

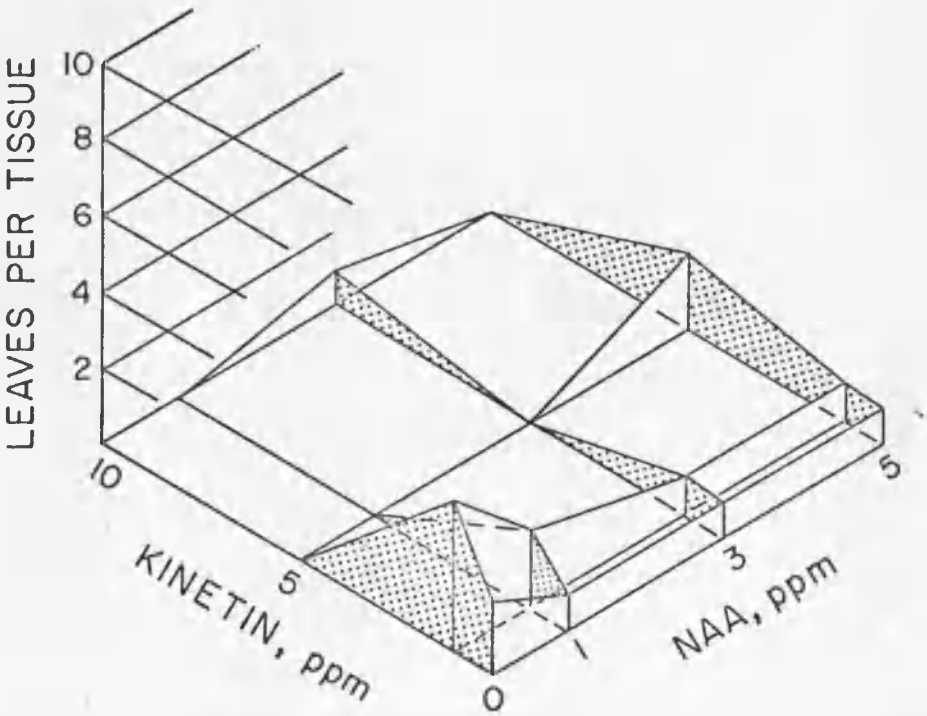


Figure 14. Number of Leaves Developed from Stem Segments as Influenced by 1-10 ppm Kinetin and 1-5 ppm NAA Added to the Medium Alone and in Combination.

Figure 15. Three Week Old Plantlet with Well Developed Root System and Poor Shoot Which Formed After Transferring Callus Initiated on a Medium Containing 1 ppm Kinetin plus 1 ppm NAA to the Control.

Figure 16. Two Week Old Plantlet Developed After Transferring Callus, with Shoot, Initiated on a Medium Containing 1 ppm Kinetin Plus 5 ppm NAA to a Medium Supplemented with 1 ppm Kinetin Plus 5 ppm IBA.


Figure 15. Plantlet with Well Developed Root and Poor Shoot

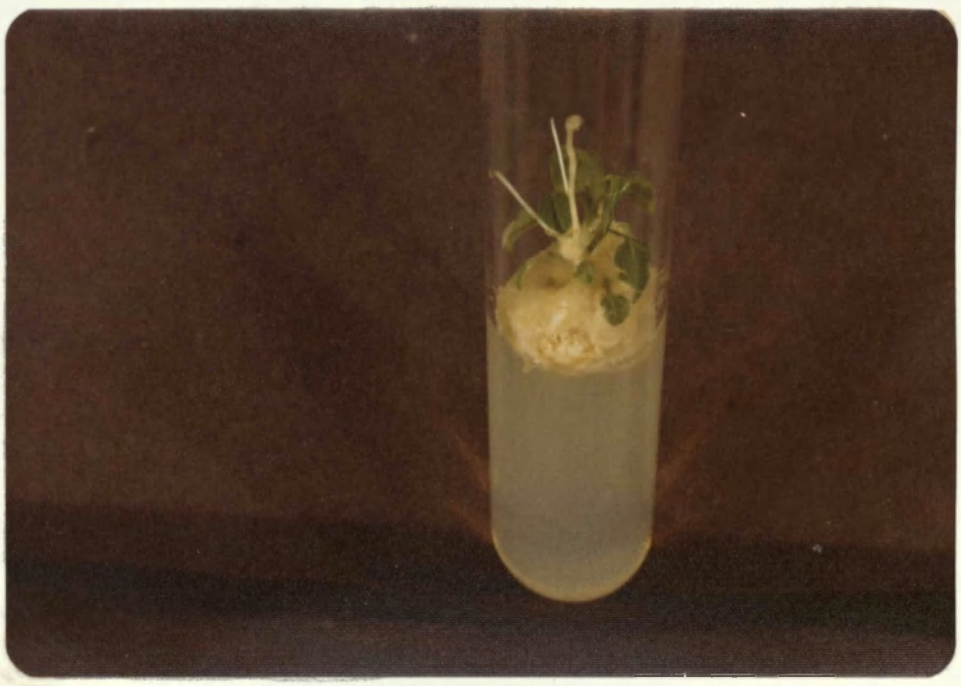

Figure 16. Plantlet from Medium with Kinetin plus NAA

The only other treatment which produced complete plantlets in a percentile of 20 was the one which was previously treated with 1 ppm kinetin plus 5 ppm NAA and transferred to a medium with 1 ppm kinetin plus 5 ppm IBA (Fig. 16).

## DISCUSSION AND CONCLUSIONS

The results of this thesis demonstrate that shoot and root formation can be chemically induced on cultured papaya shoot tips. Both auxin and kinetin were required for growth and the particular type of growth depended on their concentrations, whether used alone or in combination. Auxin appeared to be essential for callus and root formation. Kinetin, on the other hand, had a specific effect inducing shoot formation especially at a level of 1 ppm. In interaction with this concentration of kinetin, both NAA and IBA gave maximum callus production at 5 ppm and IAA at 10 ppm. Of the three auxins examined, IBA was the most effective in giving highest growth values and inducing root formation. From the results it cannot be unequivocally judged, however, whether this auxin was superior to the rest of the tested ones due to stability, presistance, or just to its specific physiological action on cultured papaya shoot tips.

In general, all the treatments used on papaya stem segments resulted in poor growth values and leaf production in contrast to shoot tips. It might be possible, however, to obtain better results with excised stem segments by using

other kinds of growth regulators and utilizing a wider range of concentrations than those already tested.

The response of papaya cultured shoot tips to kinetins and auxins was in general agreement with results obtained on plant tissue culture. A relatively high auxin/low kinetin balance stimulated rooting. Shoots, on the other hand, were produced by using low auxin/high kinetin balance, except in cases where kinetin was used in levels above 1 ppm where it showed some toxic effects on the tissues. Transferring shoots which had been induced to form on treatments with the control and the medium containing 1 ppm kinetin to a medium with 1 ppm kinetin plus 5 ppm IBA induced callus formation followed by rooting. Thus, roots were induced to form when previously differentiated shoots were transferred to a medium containing more auxin and less kinetin, i.e., when proportions required for shoot formation were reversed. It also should be noted that tissue retained on the same media did not root, indicating that transfer affected papaya tissue in a positive way. Medium containing 1 ppm kinetin plus 5 ppm IBA was selected as the best for the formation of plantlets after transferring previously formed shoots to it.

Figures 9 and 10 show the difference between the plantlets so developed. This demonstrates that the addition

of 1 ppm kinetin to the medium which preceeded the transfer of the shoots had a substantial effect on the plantlets' vigor.

A majority of the induced roots were negatively geotropic and grew away from the callus mass into the air (Figs. 9 and 16). High light intensity used in the experiment may have been a factor in reversing the normal geotropic response of those roots. The results of this experiment, however, does not permit one to draw specific conclusions on the effect of light since no study was made on the influence of this factor. Other factors may include the unequal distribution of growth regulators in the plantlets. Different levels of growth regulators tested, however, had no effect on the geotropic response of the roots since plantlets formed from the same treatments gave both negatively geotropic and normal roots (Fig. 9). Further study is needed to overcome this phenomenon.

Another factor which should be considered of significant importance is that roots growing into the medium were like "water roots," devoid of root hairs (Fig. 10). Some investigators reported difficulty in establishing such plants when removed from the culture medium and planted in soil (Pillai and Hildebrandt, 1969b). If further studies are

conducted it is suggested that either fiber-glass or filter paper wick technique could be worth testing.

Some physiological studies indicate that translocation of manufactured cytokinins from the roots to the shoot is important for the latter's growth. The results of this study were in agreement with the above. Shoots of rooted plantlets were more vigorous in their growth than nonrooted ones. A possible explanation for this observation might be attributed to the reason already mentioned. Other possibilities include: (1) growth promoting substances which had been concentrated for root differentiation and their subsequent growth are now being deflected to the shoot; (2) the more efficient absorption of nutrients and growth regulators after the root formation. The above possibilities might work separately or in combination. This remains a problem of detail, and further studies are needed to determine the exact causes.

An interesting phenomenon observed in this study was the differentiation and formation of flower-like structures on the piece of callus shown in Figure 17. They started to form after a piece of callus initiated on a medium supplemented with 1 ppm kinetin plus 5 ppm IBA was transferred for the second time on the same media. Disappearance of the chlorophyll pigment of the leaves accompanied the formation

Figure 17. Development of Flower-like Structures from the Callus.

of such structures. Such an occurrence might be attributed to the complex interaction of exogenous and/or endogenous growth regulating substances. Other factors might be involved which cannot be determined on the basis of this study.

The principal objective of this study was to select the proper medium to be a key for propagating papaya plants by means of tissue culture. Refinement of nutrient media, culture conditions, and transplanting steps are still to be achieved before large scale practical application of this method can be realized. Another area to be explored is trying to culture tissues taken from mature plants which may open a new avenue to a better way of clonal propagation of this species.

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