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TISSUE CULTURE OF PAPAYA (CARICA PAPAYA L.)

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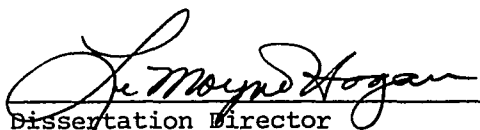
Ali Ahmed Al-Mehdi

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN HORTICULTURE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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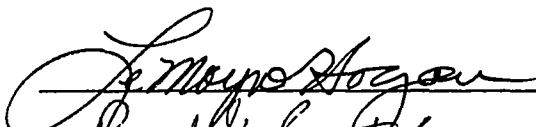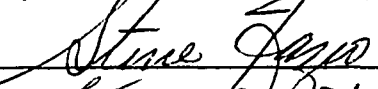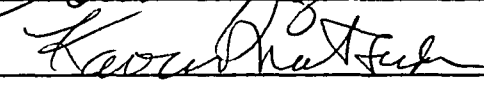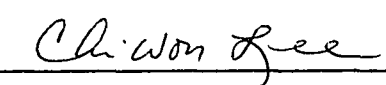
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction

by _____ Ali Ahmed Al-Mehdi _____

entitled _____ Tissue Culture of Papaya (Carica papaya L.) and _____

_____ Date Palm (Phoenix dactylifera L.) _____

be accepted as fulfilling the dissertation requirement for the Degree

of _____ Doctor of Philosophy _____.

_____   7/11/79
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As members of the Final Examination Committee, we certify that we have

read this dissertation and agree that it may be presented for final

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Final approval and acceptance of this dissertation is contingent on the
candidate's adequate performance and defense thereof at the final oral
examination.

11/78

STATEMENT BY AUTHOR

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ACKNOWLEDGMENT

The author wishes to express sincere appreciation and gratitude to his major professor, Dr. LeMoyne Hogan, for his financial assistance and encouragement during this study.

Appreciation and sincere thanks are extended to the members of the guidance committee Dr. Kaoru Matsuda, Dr. Paul Bartels, Dr. Chi Won Lee, and Professor Steve Fazio, for their critical review of this manuscript.

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ABSTRACT

The potential propagation of papaya (Carica papaya L. var. Solo Line 8) and date palm (Phoenix dactylifera L. var. Medjool) was explored.

Multiple papaya plantlets from each shoot tip excised from seedling and adult plants and seedling internodal section were obtained using a simplified Yie and Liaw's (YL) medium. Each shoot tip excised from seedling and adult plants cultured on a simplified Murashige and Skoog's (MS) medium yielded an individual plantlet. Tissue culture-derived plants were established in soil in a greenhouse with a survival rate of 85%.

The simplification of both MS and YL media was accomplished by the deletion of glycine, myo-inositol, nicotinic acid, pyridoxine, and boric acid and casein hydrolysate from the MS medium. The deletion of these substances resulted in little or no stimulation to either growth or organogenesis of the cultured papaya explants. Among the nutrient factors, thiamine was necessary for the initiation and growth of roots. Adenine was essential for initiation and growth of shoots but was suppressive to rooting.

Excised embryos, male flowers, anthers, and buds of date palm were cultured on the complete MS medium containing 2 g/l polyvinyl pyrrolidone (PVP) and different types and concentrations of growth regulators. Excised embryos germinated when IBA and kinetin were added to the medium. The perianths of the male flowers greened when 1 mg/l

kinetin was added to the medium. All cultured buds, male flowers and anthers deteriorated and eventually died due to their discharge to the medium of a brown substance.

INTRODUCTION

Papaya (<u>Carica</u> <u>papaya</u> L.) plants can have either staminate, pistillate or polygamous flowers. Presently, papaya is propagated primarily by seed and even small plantations have individuals that differ widely in sex expression and fruit characters. This variability, which reduces yield and quality, can be overcome if satisfactory methods are developed for asexual propagation of plants with desirable traits.

There have been several attempts to propagate papaya plants through cuttings (Allan, 1964; Sen Gupta and Chattopadhaya, 1954; Traub and Marshall, 1937) and grafting (Sookmark and Tai, 1975; Lang, 1969; Riccelli, 1963; Jimenez, 1959; Hancock, 1940) but these have not been commercially feasible. A high percentage of rotting of stem cuttings has made this method impractical when carried out in a large scale operation. Also since papaya has a non-branching growth habit, the amount of stem cutting materials is limited. Decapitation of the unbranched stem may stimulate one to three dormant axillary buds to form branches but poor fruit production and sex reversal commonly result from such practice. Grafting has not been successful due to the hollow nature of the papaya stem which makes it vulnerable to breakage in windy conditions.

The first attempt to culture papaya organs and/or tissues <u>in</u> <u>vitro</u> was made by Phadnis, Budrukkar and Kaulgud (1970) who successfully cultured mature embryos in White's medium. The plants established from

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embryo culture showed the same problem of genetic variability associated with seed propagation. A second attempt was made by Medora, Campbell and Mell (1973). They were successful in inducing callus by culturing 1½ inch tall seedlings on modified White and Risser's medium. Unfortunately, no attempts were made by Medora and his associates to differentiate the callus into plantlets since their intent was merely to prove the presence of proteolytic enzyme activity in papaya callus. Al-Mehdi (1976) established single plantlets from shoot tips taken from month-old seedlings. The largest number of complete plantlets were obtained after transferring shoots which were formed on Murashige and Skoog's (MS) medium supplemented with kinetin at 1 mg/l to the medium containing kinetin at 1 mg/l plus indole-3-butyric acid (IBA) at 5 mg/l. Yie and Liaw (1977) succeeded in differentiating multiple plantlets from both internodal sections and shoot tips using seedlings as the explant source. Litz and Conover (1978) were able to develop plantlets by culturing shoot tips of mature field grown plants on MS medium. Arora and Singh (1978a) could not differentiate the lateral buds, stem and leaf segments, excised from mature plants, into plantlets. The main obstacle was the secretion of latex from the cut end of the explants. Arora and Singh (1978b) were able, however, to develop callus from stem segments excised from young seedlings when cultured on MS medium with naphthaleneacetic acid (NAA).

Earle and Langhans (1974) believe that before the tissue culture technique can be seriously considered as an alternative to the conventional methods of propagation of any existing cultivar, the following requirements should be met: (1) culture can be consistently

established from defined and readily available plant parts; (2) culture can produce plantlets under defined conditions; (3) plantlets can be successfully transplanted to greenhouse and field; (4) ability to re-organize is retained after repeated subculturing; (5) plantlets develop into plants like the parents, both initially and after repeated sub-culturing; (6) rates of production are comparable to or better than rates for conventional propagation; (7) the system works for different cultivars without drastic modifications; (8) culture can be stored with minimum of care; (9) transfer and manipulation required per plantlet produced is not excessive.

All methods published to date concerning the in vitro propaga-tion of papaya have not resolved some of the basic problems which currently prohibit large scale practical application. The objectives of this portion of study on papaya are to: (1) establish aseptic cul-tures; (2) determine if Murashige and Skoog's (MS) or Yie and Liaw's (YL) media can be simplified; (3) seek cultures in which plantlets can be regenerated to eliminate the need to transfer developing explants into another medium; (4) culture shoot tips of mature plants; and (5) establish in vitro propagated plants in soil.

Date-palm (Phoenix dactylifera L.) is a dioecious plant and both staminate and pistillate plants are normally propagated by off-shoots which develop from axillary buds on the trunk. The strict dependence for propagating date-palm trees by offshoots is due to several problems associated with seed propagation. About half of seed-ling palms are males and two good palms usually produce enough pollen for fifty females. Thus, yields can be increased considerably by

planting mostly pistillate trees. Also palms derived from seeds are very heterogenous and relatively few of them produce fruit of good quality. These two factors have restricted the use of seed for breeding purposes. When a seedling palm appears outstanding in any way, it can be propagated by its offshoots, which will generally reproduce the parent type; then it becomes essentially a new cultivar.

Propagating a date-palm via offshoots also creates problems. Obtaining offshoots in sufficient number for commercial propagation is very slow. Attempts by Reuveni, Adato and Lilien-Kipnis (1972) to increase the number of offshoots produced by individual palms through rejuvenation were not successful. Additionally, the large size of offshoots, which can weigh up to 40 pounds, and the cost of transportation, maintenance, inspection and handling have discouraged many farmers from starting new date-palm orchards.

The successful use of tissue culture to propagate the date-palm should provide several advantages: (1) a large number of disease-free plants may be obtained from a given palm; (2) the movement of plants from one country or region to another without excessive quarantine pressure can be facilitated; and (3) cultivation of plants in early stages could be performed in small areas for convenience of observation and maintenance and plants propagated under these conditions could then be transported by air at very low cost.

Reuveni and Lilien-Kipnis (1974) attempted to culture in vitro various date-palm organs and tissues. The organs and tissues used in their studies were buds, tips (which consisted of the apical meristem and many primordial leaves surrounding it), mantle meristem, primordial

leaves, tissue-removed leaf bases and leaf sections-all taken from young offshoots; cotyledonary sheath sections and growing points taken from in vitro-germinated seedlings; whole embryos; fruit stalk, ovaries, unfertilized ovules and partheno-genetic endosperm; and roots. Except for excised embryos, none of the above tissues gave satisfactory results. The main problem causing failure of the explants to differentiate to plantlets was the browning of the tissues and organs, except for excised embryos, coming from the oxidation of polyphenols present in the tissues. All attempts to prevent tissue browning were unsuccessful.

The objective of this portion of study on date-palm was to find a new and rapid method for the propagation of date-palms using tissue and organ culture technique. In an attempt to accomplish this objective: (1) buds present in the axils of the primordial leaves between the spike leaf and the growing point of five year-old offshoots were cultured on the complete MS medium; (2) the effect of the addition of 0 to 10 mg/l each of kinetin and IBA to the complete MS medium on the growth and development of excised embryos was determined; and (3) whole male flowers and excised anthers were cultured on the complete MS medium.

LITERATURE REVIEW

There are numerous examples of plant regeneration from cultured explants on nutrient media but specific conditions must be determined for each plant species.

The importance of nutrient laden media is that excised plant parts soon deplete the nourishment in their own tissues unless supplied with a new source. Media of varying composition have been prepared, and these have often been modified or simplified in order to satisfy the nutrient requirement of a particular plant material. Murashige and Skoog (1962) noticed that when their medium was used for cultivation of various species and strains of tobacco, the results varied from very good growth to no growth at all. Hence, they suggested that their medium should be modified to accommodate the need of a given tissue.

Eeuwens (1976) developed a new mineral formulation, which he called Y3, on which he found that the growth of stem, leaf, and inflorescence explants of the coconut palm (Cocos nucifera) was superior to that on the minerals of White, Heller, or Murashige and Skoog. He concluded that growth of the coconut palm explants on White's and Heller's minerals was seriously limited by deficiencies in nitrogen (particularly ammonium), potassium, phosphorus, iron, iodine and molybdenum. He also concluded that the Murashige and Skoog's formulation was deficient only in certain micro-elements (particularly iodine).

A medium for optimal growth of excised coconut embryos was developed by Fisher and Tsai (1978). The liquid basal MS medium

supplemented with coconut milk, IAA and 2IP was used. A single callus

line was initiated but attempts to induce organogenesis in the callus

were unsuccessful.

A common problem of several plants that has also plagued the

successful culture of palm tissue and organs is their discharge of

phenolic compounds into the medium, commonly called "browning". This

problem becomes intensified if the tissues and organs are injured during

the excision process. Rabechault, Ahee and Guenin (1976) studied the

effect of different growth regulators on browning of excised oil palm

embryos. They noted that indole-3-acetic acid (IAA), NAA, 2,4-

dichlorophenoxy acetic acid (2,4-D) and kinetin at concentrations above

10^{-8}M caused increase tissue and media browning. Reuveni and Lilien-

Kipnis (1974) cultured several date-palm tissues and organs on MS

medium. The main problem which Reuveni and his associate encountered

was browning of the cultured tissues and organs and which caused

their growth inhibition and eventual death. Reuveni and colleagues

attempted to prevent this problem by amending the medium with acti-

vated charcoal, ascorbic acid, citric acid and polyvinyl pyrrolidone

(PVP); however, all methods were unsuccessful. The effectiveness of

these compounds in preventing tissue browning seems dependent on the

type of plant species from which the explants are taken. Christiansen

and Fonnebech (1975), for example, eliminated browning of cultured

Hamamelis intermedia shoot tips by adding 1% PVP to the medium.

The most critical organic components of plant tissue culture

media are the growth regulators. There are two major classes of plant

growth regulators which have been widely used in plant tissue culture researches: auxins and cytokinins. Selecting a particular type of auxin or cytokinin depends on the result of its physiological action, stability, persistence, and mobility. Some tissue culturists have preferred the use of the synthetic auxins IBA, NAA, or 2,4-D over the natural occurring one, IAA, due to their greater chemical stability and low mobility. On the subject of auxin mobility Kulesche (1972) working with Jerusalem artichoke showed that IAA, NAA, and 2,4-D were incorporated in the cultured tissues after 1,2-3, and 48 hours respectively. Greater chemical stability of any growth regulator provides improved persistence 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. Pennazio (1973) confirmed the conclusion that explants of some species contain sufficient endogenous levels of growth substances to support their growth in vitro into complete plants by successfully producing carnations by means of shoot tip culture without the addition of growth regulators to his medium.

A tissue culture can be started from a variety of plant parts; however, selecting the proper explant is a major governing factor in the success in obtaining plant regeneration in vitro.

Culture of flowers and flower parts have been used by several researchers to obtain plants in vitro: chrysanthemum -- sections of pedicels (Roset and Bokelmann, 1975), freesia -- flower buds (Pierick

and Steegmans, 1975), day lilies -- inflorescence slices (Meyer, 1976), broccoli -- flower curds (Anderson, Meagher and Nelson, 1977), and onion -- receptacle (Matsubara and Hihara, 1978).

Stewart and Button (1975) found different plant parts varies widely in their ability to differentiate into plantlets. Cultured explants of inflorescence stalk, leaf tips, root tips and stamens of Paphiopedilum did not show any sign of growth. The placenta of ovary sections continued to grow and differentiate, but no callus proliferation occurred. However, callus which subsequently gave rise to plantlets, could be differentiated from cultured stem apices.

There are two terms that have been used interchangeably thus causing some confusion -- shoot tip and apical meristem cultures. This confusion in the literature has resulted from the failure of several tissue culturists to report the dimensions of their explants. True meristem culture, i.e., cultures in which the explants are limited to the apical dome and measuring 0.1-1 mm in height, are not used frequently. Such cultures are valuable in morphogenetic investigations concerned with organogenesis in the shoot tip region, and additionally they are often free of pathogens, including viruses and viroids, but the frequency of successful plant development may be lower. Shoot tips, however, should be used for rapid clonal propagation. Shoot tip cultures normally start with apical segments of 1 to 10 mm in length, and occasionally longer. These large explants often multiply rapidly but do not necessarily exclude viruses or viroids that may already be infecting the plant. Before culturing these shoot tips on nutrient media, proper treatments are required to eliminate any pathogens.

Among horticultural crops in which plants have been produced recently through the _in vitro_ culture of their shoot tips or apical meristems include: <u>Aechma fasciata</u> and other bromiliades (Jones and Murashige, 1974), hop (Adams, 1975), virus-free narcissus (Mowat and Chambers, 1975), brussels sprout (Dunwell and Davies, 1975), carnation (Earle and Langhans, 1975), the cactus <u>Opuntia polyacantha</u> (Mauseth and Halperin, 1975), freesia (Petru, Jirsakova and Landa, 1976), sour cherry (Papov, Vysotskii and Trushechkin, 1976), apple (Abbott and Whiteley, 1976), tomato (Kartha et al., 1977), the orchid <u>Disa uniflora</u> (Hass, 1977), Welsh onion (Fujieda, Ando and Fujieda, 1977), almond (Tabachnik and Kester, 1977), <u>Bougainvillea glabra</u> (Chaturverdi, Sharma and Prasad, 1978), cauliflower (Grout and Aston, 1978), lettuce (Koevary, Rappaport and Morris, 1978), cucumber (Rute, Butenko and Maurinya, 1978), and blackberry (Broome and Zimmerman, 1978).

Several tissue culturists have chosen stem sections as initial explants and were able to obtain plantlet generation _in vitro_. Horticultural crops in which plants have been produced through _in vitro_ culture of their stem sections include: poinsettia (Langhe, Debergh and Van Rijk, 1974), rape (Kartha, Gamborg and Constabel, 1974), <u>Prunus amygdalus</u> (Mehra and Mehra, 1974), <u>Citrus sinensis</u> (Chaturverdi and Mitra, 1974), red cabbage (Bajaj and Nietsch, 1975), asparagus (Yang and Clore, 1975), <u>Cordyline terminalis</u> (Kunisaki, 1975), <u>Dracaena deremensis</u> cv. Warnecki (Debergh, 1975), <u>Petunia inflata</u> cv. Cascade and <u>P. hybrida</u> cv. Rose du Ciel (Sangwan and Harada, 1976), geranium (Skirvin and Janick, 1976), tomato (Langhe and de Bruijne, 1976),

Avocado (Schroeder, 1976), Dioscorea floribunda (Sita, Bammi and Randhawa, 1976), and a french hybrid of grape (Krul and Worley, 1977).

Recent researchers have focused on the attainment of haploid plants through cultures of anthers containing young microspores. Some successes have been achieved with microspores that have been separated from the anthers; but in most instances plant development from microspores occurs only when the microspores are still contained within the explanted anther. Haploid plants are attracting considerable interest among geneticists and plant hybridizers because mutants can be formed more easily in haploids than in diploids.

The developmental stage of the microspores at excision is the most critical requirement for success in anther cultures. The optimum stage occurs somewhere between the tetrad stage of the microspores and just past the first pollen mitosis (Nitsch, 1972).

Embryoid initiation from microspores apparently occurs from the vegetative, not the generative, cell (Sunderland and Wicks, 1971). Thus, an important consideration is to provide conditions that favor formation of vegetative cells at the expense of generative cells. Exposure of flower buds to low temperatures for prescribed periods (3-5° C, 48-72 hours) seems to direct the first division of the pollen nucleous toward the formation of two vegetative cells, instead of one each of vegetative and generative cells. This increases the frequency of embryogenic microspores (Malhotra and Maheshwari, 1977). There are exceptions as for example in henbane (Hyoscyamus niger) the embryoids originate predominantly from the generative cells (Raghavan, 1976).

Among crops where plants have been produced through the _in vitro_ culture of their anthers include: alfalfa (Saunders and Bingham, 1972), african violet (Hughes, Bell and Caponetti, 1975), and strawberry (Rosati, Devereux and Laneri, 1975).

MATERIALS AND METHODS

Source of Explants

Papaya

Papaya (Carica papaya L. var. Solo Line 8) seeds, supplied through the courtesy of Dr. Hamilton of the University of Hawaii, were germinated in flats in a greenhouse under intermittent misting (10 sec/ 2 min). Temperatures in the greenhouse ranged from approximately $26^{o}C$ during the day to $21^{o}C$ during the night. Plants reaching the age of 4 months were transferred to 1 gallon pots and at 6 months were transferred again to 5 gallon pots. A fertilizer of an analysis 20-20-20 (Peter Co., Allentown, Pennsylvania) was applied once every two weeks in an amount of one tablespoonful per gallon of water. Two month-old seedlings and two year-old plants were used as the source of explants. In order to increase the number of available explants, the terminal buds were removed from some of the two year-old plants. Two to three dormant axillary buds were stimulated to grow by this procedure.

Date Palm

Date-palm (Phoenix dactylifera L. var Halawy) seeds, supplied through the courtesy of the Ministry of Higher Eduction and Scientific Research, Baghdad, Iraq, were used for the in vitro culture of excised embryos. Male spathes of P. dactylifera var. Medjool, supplied by the University of Arizona Citrus Experiment Station, Mesa, Arizona, were used for the in vitro culture of excised anthers and whole male flowers.

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Tissue Excision and Sterilization

Papaya

Five millimeter-long shoot tips (weighing 3 ± 0.5 mg each) and 3 mm-long internodal sections (weighing 5 ± 0.05 mg each) were excised from 2 month-old seedlings by using a razor blade. Larger petioles, and in some cases flower primordia, were removed in order to excise 5 mm long terminal and lateral tips (weighing 15 ± 3 mg each) from the 2 year-old plants. Excision was accomplished under a low-power binocular dissecting microscope (magnification x 15) and infected and injured explants were discarded. Explants were surface sterilized by immersing in 10% v/v household bleach,[1] diluted with distilled water, for 20 minutes then rinsed three times with sterile distilled water.[2]

Date Palm

Seeds were surface scarified with sand paper, surface sterilized by immersing in 10% v/v household bleach for 2 hours, rinsed four times with sterile distilled water and finally, to soften the endosperm, soaked in sterile distilled water for six days. Embryos were excised aseptically in a sterile glass-fronted hood as follows: The seed was cut longitudinally a little to the side of the suture; holding the larger embryo containing half-seed with one-hand, the embryo was prised out of

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1. Household bleach contains 5.25% sodium hypochlorite as the active ingredient.

2. Sterility of the distilled water was accomplished by autoclaving for 20 minutes under 15 lb/inch^2 (1.1 kg/cm^2) at 240° F (116°C).

the endosperm using a fixed blade scalpel; the embryo was immersed in 2% v/v amphyl for 1 minute and rinsed three times with sterile distilled water.

Unopened male spathes were first kept in a cold room at 4°C for 48 hours and then opened in a sterile glass-fronted hood. Flower buds were removed singly, surface sterilized by immersing in 5% v/v household bleach for 5 minutes and finally rinsed three times with sterile distilled water. Anthers were removed, in a sterile glass-fronted hood, from the surface-sterilized flower buds and cultured without any further sterilization procedures.

The detailed procedure for surface sterilization of both papaya and date-palm explants was as follows: After a sufficient number of explants were obtained, they were wrapped in 10 cm squares of fine nylon mesh, transferred to 125 ml Erlenmyer flask containing a generous quantity of household bleach at the required concentration; the flask was capped with aluminum foil and constantly agitated for the required time by placing it on a Brunswick shaker (100 rpm). The explants were then rinsed three times with sterile distilled water.

Types of Media Used

Papaya

The culture of shoot tips excised from 2 month-old seedlings and 2 year-old plants was performed on Murashige and Skoog's (1962) medium as well as Yie and Liaw's (1977) medium. Internodal sections excised from the upper half of shoots of 2 month-old seedlings were cultured only on YL medium. The media of MS and YL both contain a large number

of additives. In order to determine if growth and development of papaya
explants can occur in simplified media, the omission of glycine, myo-
inositol, thiamine.HCl and nicotinic acid from both of the aforemen-
tioned media, in addition to the omission of casein acid hydrolysate
from MS medium and adenine from YL medium, was done either singly or in
combination. As a part of the studies, cultures were also made with MS
and YL media in which pyridoxine.HCl and boric acid were omitted
either singly or in combination.

In all cases, except for changes in the growth regulators shown
in Table 1, the above MS and YL media treatments were maintained constant
from the primary culture to all subsequent transfers.

Table 1. Types and concentrations of growth regulators used to culture
papaya seedling explants on YL and MS media treatments.

Type of Medium	Type of Explant	Primary Culture Media	First Transfer Media	Second Transfer Media
YL	shoot tips	0.05 mg/1 IAA + 5 mg/1 kinetin	5 mg/1 IAA	No second transfer was employed
YL	internodal sections	1 mg/1 NAA + 0.1 mg/1 kinetin	2 mg/1 kinetin	5 mg/1 IAA
MS	shoot tips	1 mg/1 kinetin	5 mg/1 IBA + 1 mg/1 kinetin	No second transfer was employed

All living tissues were routinely transferred to fresh media
after five weeks. In some cases, seedling explants were maintained for
more than five weeks.

The YL and MS media treatment which produced the best growth and differentiation of seedling shoot tips were also employed to culture shoot tips obtained from 2 year-old plants.

Date Palm

Complete MS medium, supplemented with 2 g/l PVP (MW 10000) was used. Culture of excised embryos was performed on the medium containing 0 to 10 mg/l each of kinetin and IBA. For the culture of anthers and whole male flowers, 0 to 5 mg/l kinetin was added singly and in combination with 0 to 5 mg/l NAA and 0 to 10 mg/l IAA.

All growth regulators were first dissolved in three drops of 0.3 N KOH before adding them to the media-preparation flasks prior to autoclaving. All culture media were solidified by the addition of 9.2 g/l Difco Bacto-agar. Demineralized water was used for all media preparations. Media were either used on the same day or kept refrigerated at 4°C for later use.

Media Sterilization and pH Adjustment

Generally, all media were autoclaved for 17 minutes under 15 lb/inch^2 (1.1 kg/cm^2) pressure at 240°F (116°C) but coconut milk (Gibco, Grand Island, N.Y.) for the YL medium was sterilized by filtering through 0.45 μm millipore filter-type HA (Millipore Corporation, Bedford, Mass.). The filter sterilized coconut milk was then added to the autoclaved portion of the medium just before gelation. The medium was then mixed thoroughly and cooled rapidly.

The pH of all media was adjusted to 5.6 ± 0.1 with a few drops of either 1N KOH or 1N HCl prior to autoclaving. Occasional tests

revealed that autoclaving had no significant effect on the pH of the media. The pH of the coconut milk was also adjusted to 5.6 ± 0.1 prior to filter sterilization.

Tissue Transfer to Culture Vessels

Pyrex culture tubes (2.5 x 15 cm), containing 25 ml of media, were used to culture papaya explants and date-palm embryos. Screw-cap culture bottles (3 x 6.5 cm), containing 10 ml of media were used to culture date-palm anthers and whole male flowers. The bakelite screw-caps were only lightly tightened during autoclaving and tissue incubation periods. Polypropylene slip-over closures (Bellco kap-uts) were used to cap the 2.5 x 15 cm culture tubes.

Transfer of explants to the culture vessels was performed in a glass-fronted hood. Sterility in the hood was maintained by UV light and washing with both 95% ethyl alcohol and 2% amphyl solutions.

Papaya shoot tips and date palm male flowers were placed apical-side-up while papaya internodal sections and date-palm anthers were placed horizontally on the agar-nutrient media. All explants were anchored by immersing their lower half in the media. Their positions were checked by a head gear optical glass binocular magnifier and any necessary reorientations were made with a sterilized round tipped needle.

Culture Environment

Date-palm and papaya explants cultured on the MS medium were incubated at a constant temperature of 27 ± 2°C and light intensity of 5000 lux. The cultures were illuminated 8 hours daily. Environments used to incubate papaya explants cultured on the YL medium were kept at

a constant temperature of 27 ± 2°C and light intensity of 1250 lux.
These explants were illuminated 15 hours daily.

No humidity control was used in the growth room, but instead,
cultures placed in racks were covered with clear plastic bags to lessen
the amount of evaporation from the media. Light intensity was measured
at the level of the tissue in the culture vessels. Light was emitted
from Westinghouse 40-W cool white fluorescent tubes. Whenever lower
light intensity than that available in the growth room was required,
layers of clear plastic were placed on the cultures until the needed
light intensity was attained. Culture vessels were occasionally rotated
to eliminate any bias due to temperature and light differences in the
growth room.

Histology

Histological sections of papaya tissues were prepared according
to Berlyn and Miksche (1976). The tissues were fixed in FAA (95% ethyl
alcohol: glacial acetic acid: 40% formaldehyde: distilled water, 10:1:
2:7, v:v:v:v), dehydrated through the tertiary butyl alcohol (TBA)
series and embedded in paraffin. Using OAC microtome-model 815, ten
μm thick sections were made. The sections were affixed to slides,
stained with safranin, orange G and fast green dyes and examined under
the microscope. Photographs were taken with Kodak Photomicrography
Color Film 2483 in an AO-Spencer trinocular microscope.

To determine the stage of development of the date-palm male
spathe and the location therein of the flowers which would yield a high
percentage of pollen at the uninucleate and binucleate stages, five

flower strands were removed from each male spathe and fixed in ethanol-acetic acid (3:1 v/v) overnight. Individual anthers taken from flowers located at different regions of the strand (upper, middle and lower portions) were squashed in a drop of propionic-carmine (propionic acid: carmine: distilled water, 90:1:110, v:v:v) dye and examined by phase contrast microscopy using an oil immersion objective.

Statistical Analysis

One explant was used per culture vessel and five replicants comprised each treatment. Growth measurement of the tissues consisted of the number of shoots, % ·shoots rooted in vitro (whenever applicable) and the ratios of the final to initial fresh weights. These ratios were labelled as growth values. All cultures, unless otherwise noted, were incubated for five weeks when measurements were taken. Analysis of variance was calculated for data concerned with the omission of some of the nutrients of MS and YL media which were used to culture papaya explants. In all cases the results presented were confirmed in one or more experiments.

RESULTS

Papaya

Disinfection of Plant Material

Explants were first disinfected by immersion in 10% household
bleach for 15 minutes but the percentage of contamination remained rela-
tively high, reaching 45% in the case of cultures of shoot tips obtained
from 2 year-old plants. Litz and Conover (1978) faced the same problem
with more than 95% of all primary explants having to be discarded. There-
fore, a more rigid surface sterilization procedure was needed. This led
to an experiment in which solutions containing 15% and 20% household
bleach were tested with immersion periods of 20-30 and 5-15 minutes,
respectively. Best results, with a contamination rate of 6%, were
achieved when 10% household bleach was used with an immersion time of 20
minutes followed by three rinses with sterile distilled water. Higher
concentrations and longer immersion periods caused growth inhibition.

Modification of MS Medium

Using shoot tips excised from 2 month-old seedlings, a study was
made to determine the effects of omitting various nutrient substances in
MS medium. Glycine, myo-inositol, casein, thiamine, and nicotinic acid
were omitted, singly as well as in combination. Data of the effects of
these five nutrients on the growth values of the tissues and the percen-
tage of rooted shoots are shown in Table 2, and the analysis of variance
of these data is presented in Table 3. Table 2 shows that the inclusion

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Table 2. Effects of various components of MS medium on the growth and development of papaya seedling shoot tips.

| Additive[a] | | | | | Growth Value of Tissues | | % Shoots Rooted |
G	I	C	T	N	Primary Culture[b]	First Transfer[c]	
+	+	+	+	+	157 ± 85	348 ± 63	80 ± 45
−	+	+	+	+	125 ± 28	303 ± 23	80 ± 45
+	−	+	+	+	111 ± 41	271 ± 13	100 ± 0
+	+	−	+	+	128 ± 23	301 ± 34	60 ± 55
+	+	+	−	+	83 ± 22	198 ± 17	40 ± 55
+	+	+	+	−	129 ± 31	269 ± 13	60 ± 55
−	−	+	+	+	116 ± 61	343 ± 24	80 ± 45
−	+	−	+	+	104 ± 44	336 ± 14	80 ± 45
−	+	+	−	+	120 ± 107	178 ± 75	20 ± 45
−	+	+	+	−	108 ± 30	260 ± 26	60 ± 55
+	−	−	+	+	92 ± 39	248 ± 38	80 ± 45
+	−	+	−	+	120 ± 48	312 ± 29	60 ± 55
+	−	+	+	−	114 ± 33	327 ± 20	60 ± 55
+	+	−	−	+	53 ± 38	289 ± 58	40 ± 55
+	+	−	+	−	119 ± 110	302 ± 38	60 ± 55
+	+	+	−	−	68 ± 54	130 ± 70	20 ± 45
−	−	−	+	+	127 ± 5	271 ± 125	80 ± 45
−	−	+	−	+	89 ± 72	163 ± 19	20 ± 45
−	−	+	+	−	96 ± 6	269 ± 42	80 ± 45

Table 2, Continued.

Additive[a]					Growth Value of Tissues		% Shoots Rooted
G	I	C	T	N	Primary Culture[b]	First Transfer[c]	
−	+	−	−	+	106 ± 16	328 ± 33	80 ± 45
−	+	−	+	−	118 ± 69	339 ± 52	60 ± 55
−	+	+	−	−	112 ± 23	281 ± 23	60 ± 55
+	−	−	−	+	73 ± 63	156 ± 78	20 ± 45
+	−	−	+	−	92 ± 10	305 ± 10	60 ± 55
+	−	+	−	−	68 ± 34	208 ± 10	20 ± 45
+	+	−	−	−	61 ± 21	186 ± 51	40 ± 55
−	−	−	−	+	77 ± 42	195 ± 134	40 ± 55
−	−	−	+	−	120 ± 10	324 ± 44	80 ± 45
−	−	+	−	−	65 ± 40	217 ± 80	40 ± 55
−	+	−	−	−	58 ± 7	162 ± 41	20 ± 45
+	−	−	−	−	83 ± 3	198 ± 34	20 ± 45
−	−	−	−	−	69 ± 13	125 ± 22	20 ± 45

[a]The letters G, I, C, T, and N refer to Glycine, Inositol, Casein, Thiamine, and Nicotinic acid, respectively. The + sign refers to additive present, the − sign refers to additive absent.

[b] and [c]These terms are defined in the Materials and Methods section.

Table 3. Analysis of variance of the effects of various components of MS medium on the growth and
development of papaya seedling shoot tips.

		Growth Values of Tissues					
		Primary Culture		First Transfer		% Shoots Rooted	
Source of Variation	DF	Mean Square	F	Mean Square	F	Mean Square	F
Main Effects	5	12,707.91	5.74*	75,434.00	27.92*	12,750.00	5.37*
Glycine	1	543.91	.25	330.63	.12	1,000.00	.42
Inositol	1	2,932.66	1.33	12,075.63	4.47	.00	.00
Casein	1	6,312.66	2.85	22.50	.01	250.00	.11
Thiamine	1	47,437.66	21.43*	346,890.63	128.37*	56,250.00	23.68*
Nicotinic Acid	1	6,312.66	2.85	17,850.63	6.61	6,250.00	2.63
2-way Interactions	10	1,194.91	.54	10,340.63	3.83*	800.00	.34
CX I	1	345.16	.16	12,425.63	4.60	250.00	.11
GXC	1	1,470.16	.66	3,240.00	1.20	1,000.00	.42
GXT	1	2,066.41	.93	1,625.63	.60	.00	.00
GXN	1	191.41	.09	525.63	.20	1,000.00	.42
IXC	1	1,856.41	.84	49,702.50	18.40*	1,000.00	.42
IXT	1	1,657.66	.75	950.63	.35	4,000.00	1.68
IXN	1	3.91	.00	20,903.63	7.75*	.00	.00
CXT	1	1,237.66	.56	1,102.50	.41	250.00	.11
CXN	1	2,287.66	1.03	122.50	.05	250.00	.11
TXN	1	832.66	.38	12,780.63	4.73	250.00	.11

Table 3, <u>Continued</u>.

		Growth Values of Tissues						
		Primary Culture			First Transfer		% Shoots Rooted	
Source of Variation	DF	Mean Square	F	Mean Square	F	Mean Square	F
3-way Interactions	10	1,990.16	.90	14,293.25	5.29*	1,600.00	.67
GXIXC	1	1,657.66	.75	2,402.50	.89	250.00	.11
GXIXT	1	14,345.16	6.48	20,025.63	7.41*	250.00	.11
GXIXN	1	3.91	.00	8,555.63	3.17	2,250.00	.95
GXCXT	1	1,925.16	.81	4,202.50	1.56	.00	.00
GXCXN	1	1,025.16	.46	16,522.50	9.82*	9,000.00	3.79
GXTXN	1	288.91	.13	9,765.63	3.61	.00	.00
IXCXT	1	68.91	.03	9,302.50	3.44	1,000.00	.42
IXCXN	1	472.66	.21	16,810.00	6.22	1,000.00	.42
IXTXN	1	113.91	.05	455.63	.17	.00	.00
CXTXN	1	.16	.00	44,890.00	16.61*	2,250.00	.95
4-way Interactions	5	1,644.16	.74	18,439.63	6.82*	1,150.00	.48
GXIXCXT	1	7.66	.00	16,000.00	5.92	250.00	.11
GXIXCXN	1	68.91	.03	2,402.50	.89	250.00	.11
GXIXTXN	1	2,363.91	1.07	1,890.63	.70	250.00	.11
GXCXTXN	1	2,932.66	1.33	55,502.50	20.539*	4,000.00	1.68
IXCXTXN	1	2,847.66	1.29	16,402.50	6.07	1,000.00	.42

Table 3, Continued.

		Growth Values of Tissues						
		Primary Culture			First Transfer		% Shoots Rooted	
Source of Variation	DF	Mean Square	F	Mean Square	F	Mean Square	F
5-way Interactions	1	26.41	.01	6,002.50	2.22	250.00	.11
GXIXCXTXN	1	26.41	.01	6,002.50	2.22	250.00	.11
Error	128	2,213.16		2,702.23		2,375.00	
Total	159	2,433.47		6,714.44		2,501.57	

* Significant at the 1% probability level.

of thiamine in the medium increased both the growth value of the tissues

and the percentage of rooted shoots. The analysis of variance showed

that thiamine alone caused highly significant differences in both the

growth value in the primary culture and the percentage of rooted shoots

(Table 3). Table 3 additionally shows that the presence of the other

tested nutrients in the medium also caused highly significant differ-

ences in the growth value of the tissues in the first transfer. The

highly significant differences to the effects on the growth value of

the tissues in the presence of nutrients other than thiamine, however,

should not be considered alone. The analysis of variance also showed

that the value of the mean square of the treatment containing thiamine

alone was higher than that of the ones containing other nutrients

(Table 3). High value of mean square of the treatment containing

thiamine alone indicates that thiamine played the most important role

of all five nutrients tested in increasing the growth values of the

tissues in the first transfer.

Data relating to the effects of pyridoxine and boric acid on

the growth value of the tissues and the percentage of rooted shoots are

presented in Table 4. Analysis of variance of these data showed that

these two substances did not significantly effect growth and development

of papaya seedling shoot tips (Tables 5 and 6).

In all cases, callus was formed at the base of the shoot tips

three days after culture. The callus was soft, friable, turgid,

and more or less transparent.

Only a single plantlet was obtained from each shoot tip excised

from seedlings (Figure 1). In an attempt to determine if multiple

Table 4. Effects of pyridoxine and boric acid on the growth and development of papaya seedling explants.

Additive*		Growth Value of Primary Culture	Growth Value of First Transfer	Number of Shoots	% Shoots Rooted
Pyridoxine	Boric Acid				
		Shoot Tips in MS Medium			
+	+	91 + 40	341 + 53		80 + 45
-	+	95 + 54	316 + 95		60 + 55
+	-	124 + 21	325 + 48		60 + 55
-	-	112 + 51	301 + 64		60 + 55
		Shoot Tips in YL Medium			
+	+	402 + 122		11.8 + 6.8	33.3 + 37.0
-	+	424 + 170		10.2 + 3.9	44.4 + 32.0
+	-	475 + 66		10.0 + 2.3	40.0 + 21.7
-	-	413 + 129		9.6 + 6.0	36.4 + 29.2
		Internodal Sections in YL Medium			
+	+	156 + 67	249 + 73	16.8 + 5.7	26.7 + 22.5
-	+	131 + 37	195 + 40	16.0 + 3.2	33.3 + 20.7
+	-	116 + 50	224 + 35	15.2 + 6.7	35.7 + 24.0
-	-	139 + 20	209 + 36	16.0 + 5.5	25.0 + 13.9

* The + sign refers to additive present, the - sign refers to additive absent.

Table 5. Analysis of variance of the effects of pyridoxine and boric acid on the growth values of papaya seedling explants.

| Source of Variation | DF | Primary Culture | | | First Transfer | |
		Mean Square	F	Mean Square	F
		Shoot Tips in MS Medium				
Main Effects	2	1,602.50	.86	2,101.25	.46
Pyridoxine	1	80.00	.04	3,001.25	.66
Boric Acid	1	3,125.00	1.67	2,101.25	.46
2-way Interactions	1	320.00	.17	1.25	.00
P x B	1	320.00	.17	1.25	.00
Error	16	1,873.13		4,573.75	
Total	19	1,762.90		4,072.83	
		Shoot Tips in YL Medium				
Main Effects	2	3,402.50	.21		
Pyridoxine	1	2,000.00	.12		
Boric Acid	1	4,805.00	.30		
2-way Interactions	1	8,820.00	.55		
P x B	1	8,820.00	.55		
Error	16	16,158.25			
Total	19	14,429.00			
		Internodal Sections in YL Medium				
Main Effects	2	1,126.25	.52	3,051.25	1.27
Pyridoxine	1	151.25	.07	5,951.25	2.49
Boric Acid	1	2,101.25	.97	151.25	.06
2-way Interaction	1	4,061.25	1.87	1,901.25	.79
P x B	1	4,061.25	1.87	1,901.25	.79
Error	16	2,177.63		2,394.25	
Total	19	2,166.09		2,437.46	

Table 6. Analysis of variance of the effect of pyridoxine and boric acid on the number of shoots and the % shoots rooted of papaya seedling explants.

Source of Variation	Number of Shoots			% Shoots Rooted	
	DF	Mean Square	F	Mean Square	F
		Shoot Tips in MS Medium			
Main Effects	2			500.00	.18
Pyridoxine	1			500.00	.18
Boric Acid	1			500.00	.18
2-way Interactions	1			500.00	.18
P x B	1			500.00	.18
Error	16			2,750.00	
Total	19			2,394.74	
		Shoot Tips in YL Medium			
Main Effects	2	5.00	.19	36.21	.04
Pyridoxine	1	5.00	.19	70.31	.08
Boric Acid	1	5.00	.19	2.11	.00
2-way Interactions	1	.00	.00	270.11	.29
P x B	1	.00	.00	270.11	.29
Error	16	25.75		930.92	
Total	19	22.21		801.96	
		Internodal Sections in YL Medium			
Main Effects	2	1.25	.04	10.81	.03
Pyridoxine	1	1.25	.04	21.01	.05
Boric Acid	1	1.25	.04	.61	.01
2-way Interactions	1	1.25	.04	374.11	.88
P x B	1	1.25	.04	374.11	.88
Error	16	29.38		426.84	
Total	19	24.93		380.27	

Figure 1. Papaya plantlet from seedling shoot tip cultured on the modified MS medium in which glycine, myo-inositol, casein, nicotinic acid, pyridoxine and boric acid were omitted.

shootlets would be formed, the terminal tip of the shoots present after the first transfer was removed aseptically in situ. In general, this did not effect the formation of multiple shoots; however, two shootlets were formed occasionally (Figure 2). Examination of these shootlets revealed that they arose as outgrowths of axillary buds.

No rooting was observed on any of the shoots that were growing for the first five weeks on the primary cultures. These shoots started senesce and media drying was observed after eight weeks in culture. Senescence of the shoots was manifested by abscission of the petioles and yellowing of the leaves. Rooting, occurred after two weeks, when these shoots were moved to the first transfer, but the percentage of shoots with roots varied from 20% to 100% depending on the composition of the medium (Table 2).

Shoot tips excised from 2 year-old plants were cultured on the primary culture medium where glycine, myo-inositol, casein hydrolysate, nicotinic acid, pyridoxine and boric acid were omitted. Sixty per cent of the developing shoots rooted when moved to the first transfer medium without added glycine, myo-inositol, casein hydrolysate, nicotinic acid, pyridoxine and boric acid. Growth values of the tissues on the primary culture and the first transfer media were 26 ± 11 and 69 ± 26, respectively. Here too, as in the case of using seedling shoot tips, only individual plantlets were obtained from each cultured shoot tip (Figure 3).

Modification of YL Medium

Shoot tips excised from 2 month-old seedlings were cultured on YL medium where glycine, myo-inositol, adenine, thiamine, and nicotinic

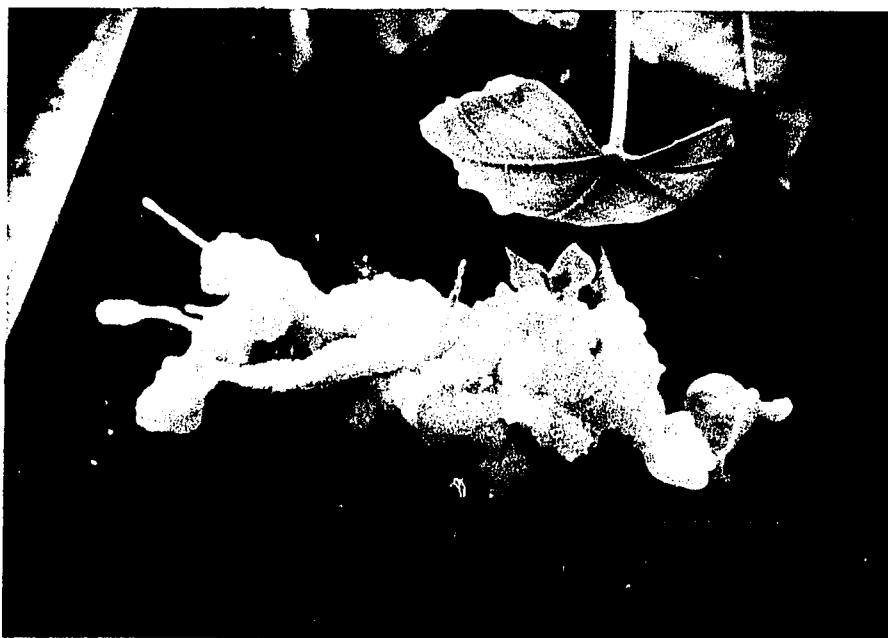Figure 2. Multiple papaya plantlet from a decapitated seedling shoot tip cultured on the modified MS medium in which glycine, myo-inositol, casein, nicotinic acid, pyridoxine and boric acid were omitted.

Figure 3. Papaya plantlet obtained from a 2 year old plant
shoot tip cultured on the modified MS medium in
which glycine, myo-inositol, casein, nicotinic
acid, pyridoxine and boric acid were omitted.

acid were excluded singly and in combination. Data of the effects of these substances on the growth value of tissues, the number of shoots, and the percentage of rooted shoots are shown in Table 7. The analysis of variance of these data is presented in Table 8. Table 7 shows that the inclusion of adenine in the medium increased the growth value of the tissues. The analysis of variance shows that highly significant differences in the growth value of the tissues resulted by adenine alone, by interactions of adenine with other tested nutrients, and by the interaction of glycine, thiamine and nicotinic acid (Table 8). The presence of adenine in the medium increased the number of shoots (Table 7). Table 8 shows that highly significant differences in the number of shoots were brought about by adenine, thiamine, and nicotinic acid alone, by the interaction of adenine and nicotinic acid, and by the interaction of glycine, myo-inositol, adenine and nicotinic acid. Table 7 shows the presence of thiamine in the medium increased the percentage of rooted shoots. Table 7 also shows that the presence of both adenine and thiamine together decreased the percentage of rooted shoots. The analysis of variance shows that highly significant differences in the percentage of rooted shoots resulted by thiamine alone and by the interaction of thiamine and adenine (Table 8). The high values of mean square of the treatments containing adenine alone indicate that this substance played the most important role of all five substances tested in increasing the growth value of the tissues and the number of shoots (Table 8). Likewise, Table 8 also shows a high value of mean square of the treatment containing thiamine alone indicating that thiamine was the

Table 7. Effects of various components of YL medium on the growth and development of papaya seedling shoot tips.

G	I	A	T	N	Growth Value of Tissue	Number of Shoots	% Shoots Rooted
+	+	+	+	+	546 ± 115	13.2 ± 4.1	38.5 ± 35.4
−	+	+	+	+	425 ± 15	11.2 ± 4.2	45.5 ± 17.7
+	−	+	+	+	417 ± 48	14.0 ± 2.3	42.9 ± 26.6
+	+	−	+	+	301 ± 33	10.0 ± 1.2	60.0 ± 21.9
+	+	+	−	+	428 ± 29	13.4 ± 2.5	30.8 ± 10.8
+	+	+	+	−	476 ± 126	14.0 ± 1.9	35.7 ± 14.3
−	−	+	+	+	427 ± 26	15.8 ± 1.6	33.3 ± 27.8
−	+	−	+	+	321 ± 44	6.6 ± 1.0	66.7 ± 23.9
−	+	+	−	+	441 ± 66	10.0 ± 8.0	20.0 ± 13.9
−	+	+	+	−	468 ± 87	14.0 ± 3.4	50.0 ± 20.7
+	−	−	+	+	351 ± 98	8.4 ± 1.9	62.7 ± 24.1
+	−	+	−	+	452 ± 48	13.6 ± 0.7	46.2 ± 34.4
+	−	+	+	−	428 ± 112	13.8 ± 3.3	46.2 ± 18.0
+	+	−	−	+	361 ± 43	5.0 ± 1.6	40.0 ± 22.7
+	+	−	+	−	309 ± 31	7.6 ± 2.9	71.4 ± 10.2
+	+	+	−	−	473 ± 113	10.0 ± 0.7	20.0 ± 14.4
−	−	−	+	+	328 ± 13	6.2 ± 1.7	66.7 ± 5.4
−	−	+	−	+	483 ± 72	13.8 ± 2.0	46.2 ± 37.1
−	−	+	+	−	471 ± 60	14.2 ± 3.8	42.9 ± 4.2

Table 7, Continued.

Additive*					Growth Value of Tissue	Number of Shoots	% Shoots Rooted
G	I	A	T	N			
−	+	−	−	+	353 ± 44	9.8 ± 3.3	22.2 ± 14.2
−	+	−	+	−	363 ± 40	5.0 ± 0.7	60.0 ± 34.8
−	+	+	−	−	464 ± 14	14.0 ± 2.5	50.0 ± 7.1
+	−	−	−	+	336 ± 41	10.0 ± 6.0	40.0 ± 31.4
+	−	−	+	−	312 ± 18	5.0 ± 2.4	60.0 ± 4.6
+	−	+	−	−	454 ± 65	10.0 ± 2.0	30.0 ± 18.0
+	+	−	−	−	344 ± 90	7.8 ± 1.2	28.6 ± 4.6
−	−	−	−	+	322 ± 15	4.0 ± 0.7	50.0 ± 34.9
−	−	−	+	−	379 ± 53	5.0 ± 3.1	60.0 ± 34.7
−	−	+	−	−	468 ± 50	12.0 ± 3.8	50.0 ± 8.2
−	+	−	−	−	296 ± 11	1.0 ± 1.4	00.0 ± 0.0
+	−	−	−	−	285 ± 70	2.0 ± 2.1	00.0 ± 0.0
−	−	−	−	−	105 ± 5	2.0 ± 1.8	50.0 ± 50.0

* The letters G, I, A, T, and N refer to Glycine, Inositol, Adenine, Thiamine, and Nicotinic Acid, respectively. The + sign refers to additive present, the − sign refers to additive absent.

Table 8. Analysis of variance of the effects of various components of YL medium on the growth and development of papaya seedling shoot tips.

Source of Variation	DF	Growth Value of Tissue Mean Square	F	Number of Shoots Mean Square	F	% Shoots Rooted Mean Square	F
Main Effects	5	166,823.91	42.42*	429.66	49.19*	3,860.63	7.19*
Glycine	1	3,950.16	1.00	26.41	3.02	705.60	1.31
Inositol	1	19,250.16	4.90	1.41	.16	1,416.10	2.64
Adenine	1	794,535.16	202.02*	1,925.16	220.41*	1,716.10	3.20
Thiamine	1	10,320.16	2.62	97.66	11.18*	15,093.23	28.12*
Nicotinic Acid	1	6,063.91	1.54	97.66	11.18*	372.10	.69
2-way Interactions	10	7,959.91	2.02	25.28	2.89*	1,483.23	2.80*
A x I	1	472.66	.12	3.91	.45	874.23	1.63
G x A	1	1,722.66	.44	56.41	6.46	112.23	.21
G x T	1	9,226.41	2.35	1.41	.161	211.60	.39
G x N	1	97.66	.03	18.91	2.17	874.23	1.63
I x C	1	1,856.41	.47	26.41	3.02	.23	.00
I x T	1	3,950.16	1.00	1.41	.161	1,716.10	3.20
I x N	1	8,337.66	2.12	35.16	4.03	378.23	.71
A x T	1	11,138.00	2.83	.156	.018	8,179.60	15.24*
A x N	1	20,588.91	5.24	82.66	9.46*	1,404.23	2.62
T x N	1	22,207.66	5.65	26.41	3.02	1,081.60	2.02
3-way Interactions	10	23,245.91	5.91*	6.78	.78	1,012.33	1.89
G x I x A	1	23,887.66	6.07	3.91	.45	3,097.60	5.77

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Table 8, Continued.

Source of Variation	DF	Growth Value of Tissue Mean Square	F	Number of Shoots Mean Square	F	% Shoots Rooted Mean Square	F
G x I x T	1	9,687.66	2.46	18.91	2.17	3,115.23	5.80
G x I x N	1	3,285.16	.04	12.66	1.45	409.60	.76
G x A x T	1	37,976.41	9.66*	1.41	.16	99.23	.19
G x A x N	1	5,581.41	1.42	18.91	2.17	883.60	1.65
G x T x N	1	41,441.41	10.54*	.16	.02	1,729.23	3.22
I x A x T	1	57,191.41	14.54*	1.41	.16	4.23	.01
I x A x N	1	8,482.66	2.16	7.66	.88	8.10	.02
I x T x N	1	15,900.16	4.04	1.41	.16	112.23	.21
A x T x N	1	29,025.16	7.38*	1.41	.16	664.23	1.24
4-way Interactions	5	987.41	.25	30.41	3.48*	531.43	.99
G x I x A x T	1	170.16	.04	3.91	.45	792.10	1.48
G x I x A x N	1	3.91	.00	82.66	9.46*	1,357.23	2.53
G x I x T x N	1	1,470.16	.37	26.41	3.02	12.10	.02
G x A x T x N	1	3,285.16	.84	35.16	4.03	19.60	.04
I x A x T x N	1	7.66	.00	3.91	.45	476.10	.89
5-way Interactions	1	11,138.91	2.83	56.41	6.46	403.23	.75
G x I x A x T x N	1	11,138.91	2.83	56.41	6.46	403.23	.75
Error	128	3,933.02		8.73		536.82	
Total	159	10,476.00		23.87		729.76	

* Significant at the 1% probability level.

major nutrient causing the highly significant increase in the percentage of rooted shoots.

Figure 4 shows a representative culture where multiple shootlets were obtained from a shoot tip cultured on the modified YL medium in which glycine, myo-inositol, thiamine and nicotinic acid where omitted. Histological studies showed that many of these shootlets arose from the initiation and growth of axillary buds (Figure 5). No rooting was observed on the shootlets for the first five weeks on any of the media compositions of the primary culture. These shootlets started to senesce and media drying was observed after eight weeks in culture. Senescence of the shootlets was manifested by abscission of the petioles and yellowing of the leaves. Rooting occurred after 15 days when these shootlets were subdivided and cultured individually (i.e., one shootlet per culture tube) on the first transfer but the percentage of shoots with roots varied from 0% to 71.4% depending on the composition of the medium (Figure 6 and Table 7).

Although not extensive, callus was formed at the base of all shoot tips and plantlets. The callus was soft, friable, turgid, and more or less transparent.

Results from the experiment in which shoot tips excised from 2 year-old plants instead of seedlings showed that 11.2 ± 2.9 shootlets, with a growth value of 93 ± 28 could be obtained from each shoot tip cultured on the primary culture where glycine, myo-inositol, thiamine, nicotinic acid, pyridoxine, and boric acid were omitted. When these shootlets were subdivided and moved individually to the first transfer where glycine, myo-inositol, adenine, nicotinic acid, pyridoxine, and
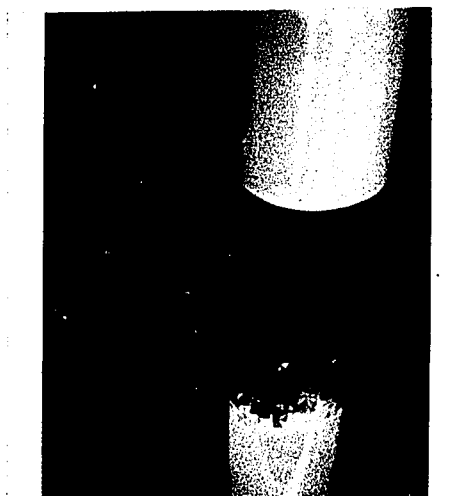
Figure 4. Multiple shootlets proliferating from papaya shoot
tip cultured on the modified YL medium in which
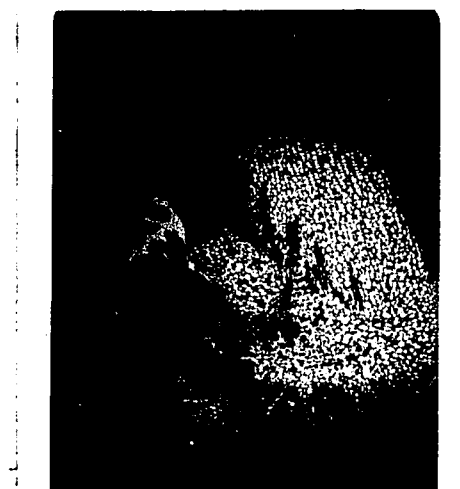glycine, myo-inositol, thiamine and nicotinic acid
were excluded.

Figure 5. Histological section of papaya shoot tip showing
the development of axillary buds. x 130.

Figure 6. Papaya plantlet obtained on the first·
          transfer of the modified YL medium.

boric acid were excluded, 54.5% ± 22.7% of these shootlets rooted. In-
terestingly enough, whole shoot tips excised from 2 year-old plants
turned into calli after four weeks on two replicant cultures of the media
composition of the primary culture where glycine, thiamine, nicotinic
acid, pyridoxine, and boric acid were omitted. When these calli were
moved at the end of the four weeks to media composition of the first
transfer where glycine, thiamine, nicotinic acid, pyridoxine, and boric
acid were omitted, they produced embryoids after three weeks in culture.
These embryoids later developed green cotyledon-like appendages and
true leaves. Attempts to reproduce these embryoids by repeating the
experiment a second time, however, were unsuccessful.

Seedling internodal sections were cultured on YL medium where
glycine, myo-inositol, adenine, thiamine, and nicotinic acid were omit-
ted singly and in combination. Data of the effects of these substances
on the growth value of the tissues, number of shoots and the percentage
of rooted shoots are presented in Table 9. The analysis of variance of
these data is presented in Tables 10 and 11. The data in Table 9 shows
that the growth value of the tissues, in both the primary culture and
the first transfer, and the number of shoots were increased by the
presence of adenine. The analysis of variance showed that highly sig-
nificant differences in both the growth value of the tissues and the
number of shoots were brought about by adenine alone (Tables 10 and 11).
Table 9 indicates that the percentage of rooted shoots decreased when
both adenine and thiamine were present together in the medium. The
analysis of variance showed that highly significant differences resulted
by the interaction of adenine and thiamine (Table 11).

Table 9. Effects of various components of YL medium on the growth and development of papaya seedling internodal sections.

Additive*					Growth Value of Tissue		Number	% Shoots Rooted
G	I	A	T	N	Primary Culture	First Transfer	of Shoots	
+	+	+	+	+	141 + 33	235 + 78	17.8 + 7.3	35.3 + 22.4
−	+	+	+	+	116 + 15	228 + 22	17.6 + 4.4	29.4 + 16.3
+	−	+	+	+	128 + 34	201 + 16	15.2 + 2.4	33.3 + 31.3
+	+	−	+	+	62 + 7	156 + 57	9.2 + 5.7	55.5 + 28.1
+	+	+	−	+	95 + 8	246 + 125	15.4 + 3.1	26.6 + 7.3
+	+	+	+	−	108 + 56	180 + 9	15.0 + 9.7	20.0 + 14.5
−	−	+	+	+	114 + 46	228 + 36	16.0 + 1.2	25.0 + 12.9
−	+	−	+	+	69 + 16	118 + 42	13.4 + 9.5	46.2 + 31.3
−	+	+	−	+	63 + 9	256 + 39	15.0 + 9.2	40.0 + 30.8
−	+	+	+	−	137 + 67	212 + 38	15.8 + 8.3	33.3 + 13.9
+	−	−	+	+	74 + 6	190 + 9	15.0 + 3.1	40.0 + 17.6
+	−	+	−	+	109 + 18	206 + 66	18.8 + 11.2	22.2 + 14.6
+	−	+	+	−	70 + 45	162 + 81	16.6 + 3.1	12.5 + 7.4
+	+	−	−	+	43 + 5	94 + 11	8.0 + 6.9	50.0 + 32.1
+	+	−	+	−	57 + 42	147 + 57	12.2 + 3.8	41.7 + 20.6
+	+	+	−	−	118 + 63	158 + 26	15.8 + 2.3	33.3 + 17.9
−	−	−	+	+	68 + 7	105 + 6	9.4 + 9.3	55.6 + 50.8
−	−	+	−	+	105 + 38	230 + 140	17.2 + 4.2	17.6 + 11.9
−	−	+	+	−	69 + 49	179 + 19	14.6 + 7.1	21.4 + 13.3

44

Table 9, Continued.

Additive*					Growth Value of Tissue		Number	% Shoots Rooted
G	I	A	T	N	Primary Culture	First Transfer	of Shoots	
−	+	−	−	+	42 ± 9	70 ± 17	7.6 ± 8.2	00.0 ± 00.0
−	+	−	+	−	47 ± 3	104 ± 26	12.8 ± 2.5	58.3 ± 36.9
−	+	+	−	−	85 ± 53	162 ± 29	12.0 ± 7.6	25.0 ± 20.5
+	−	−	−	+	60 ± 55	126 ± 132	9.2 ± 10.3	11.1 ± 12.9
+	−	−	+	−	54 ± 13	149 ± 60	8.6 ± 3.1	37.5 ± 30.1
+	−	+	−	−	103 ± 156	183 ± 17	15.2 ± 8.6	26.7 ± 30.5
+	+	−	−	−	59 ± 6	141 ± 69	7.2 ± 3.3	42.8 ± 27.1
−	−	−	−	+	64 ± 59	108 ± 21	4.0 ± 8.4	25.0 ± 43.3
−	−	−	+	−	68 ± 65	136 ± 120	5.0 ± 1.4	40.0 ± 23.5
−	−	+	−	−	112 ± 44	216 ± 151	12.2 ± 5.4	41.7 ± 28.3
−	+	−	−	−	74 ± 11	125 ± 35	7.8 ± 6.7	28.6 ± 33.9
+	−	−	−	−	59 ± 5	131 ± 32	5.0 ± 7.0	20.0 ± 19.0
−	−	−	−	−	45 ± 43	95 ± 49	5.0 ± 2.5	20.0 ± 13.2

* The letters G, I, A, T, and N refer to Glycine, Inositol, Adenine, Thiamine, and Nicotinic Acid, respectively. The + sign refers to additive present, the − sign refers to additive absent.

Table 10. Analysis of variance of the effects of various components of YL medium on the growth values of papaya seedling internodal sections.

Source of Variation	DF	Primary Culture Mean Square	F	First Transfer Mean Square	F
Main Effects	5	17,596.38	8.42*	49,602.91	11.28*
Glycine	1	600.63	.29	8,925.16	2.03
Inositol	1	30.63	.02	2,212.66	.50
Adenine	1	82,810.00	39.61*	217,931.41	49.57*
Thiamine	1	3,330.63	1.60	11,988.91	2.71
Nicotinic Acid	1	1,210.00	.58	6,956.41	1.58
2-way Interactions	10	2,380.00	1.14	5,625.78	1.28
G x I	1	225.63	.11	2,441,41	.56
G x A	1	1,000.00	.48	15,900.16	3.62
G x T	1	390.63	.19	56.41	.01
G x N	1	1,000.00	.48	5,232.66	1.19
I x A	1	1,322.50	.63	620.16	.14
I x T	1	4,515.63	2.16	5,006.41	1.4
I x N	1	6,002.50	2.87	620.16	.14
A x T	1	250.00	.12	3,106.41	.70
A x N	1	390.63	.19	18,597.66	4.23
T x N	1	8,702.50	4.16	4,676.41	1.06
3-way Interactions	10	1,195.25	.57	2,704.31	.62
G x I x A	1	640.00	.31	6,187.66	1.41

Table 10, <u>Continued</u>.

Source of Variation	DF	Primary Culture		First Transfer	
		Mean Square	F	Mean Square	F
G x I x T	1	455.63	.22	2,287.66	.52
G x I x N	1	90.00	.04	1,237.66	.28
G x A x T	1	360.00	.17	4,895.16	1.11
G x A x N	1	950.63	.46	1,025.16	.23
G x T x N	1	562.50	.27	12.66	.00
I x A x T	1	6,760.00	3.23	7,357.66	1.67
I x A x N	1	855.63	.41	2,212.66	.50
I x T x N	1	122.50	.06	472.66	.11
A x T x N	1	1,155.63	.55	1,351.41	.31
4-way Interactions	5	1,059.88	.51	3,933.91	.99
G x I x A x T	1	2,402.50	1.15	2,520.16	.57
G x I x A x N	1	140.63	.07	5,006.41	1.14
G x I x T x N	1	40.00	.02	3,285.16	.75
G x A x T x N	1	390.63	.19	3,851.41	.88
I x A x T x N	1	2,325.63	.29	5,006.41	1.14
5-way Interactions	1	2,480.63	1.19	832.66	.19
G x I x A x T x N	1	2,480.63	1.19	832.66	.18
Error	128	2,090.50		3,496.86	
Total	159	2,510.05		5,752.28	

* Significant at the 1% probability level.

Table 11. Analysis of variance of the effects of various components of YL medium on the number of shoots and the % shoots rooted of papaya seedling internodal sections.

		Number of Shoots		% Shoots Rooted	
Source of Variation	DF	Mean Square	F	Mean Square	F
Main Effects	5	456.91	10.72*	1,691.61	2.74
Glycine	1	56.41	1.32	.31	.00
Inositol	1	26.41	.62	2,117.03	3.42
Adenine	1	1,856.41	.43.55*	2,600.16	4.21
Thiamine	1	213.91	5.02	3,724.90	6.02
Nicotinic Acid	1	131.41	3.08	15.63	.03
2-way Interactions	10	27.53	.65	1,114.06	1.80
G x I	1	56.41	1.32	1,193.56	1.93
G x A	1	1.41	.03	366.03	.59
G x T	1	7.66	.18	726.76	1.18
G x N	1	1.41	.03	743.91	1.20
I x A	1	45.16	1.04	154.06	.25
I x T	1	18.91	.44	9.03	.02
I x N	1	45.16	1.06	15.63	.03
A x T	1	97.66	2.29	6,262.51	10.13*
A x N	1	1.41	.03	68.91	.11
T x N	1	.16	.00	1,600.23	2.59
3-way Interactions	10	14.53	.34	487.70	.79
G x I x A	1	35.16	.83	1,276.90	2.06

Table 11, Continued.

| Source of Variation | Number of Shoots | | | % Shoots Rooted | |
	DF	Mean Square	F	Mean Square	F
G x I x T	1	12.66	.30	985.06	1.59
G x I x N	1	12.66	.30	381.31	.62
G x A x T	1	3.91	.09	1,081.60	1.75
G x A x N	1	26.41	.62	.03	.00
G x T x N	1	3.91	.09	135.06	.22
I x A x T	1	3.91	.09	104.01	.17
I x A x N	1	26.41	.62	522.01	.24
I x T x N	1	1.41	.03	372.00	.61
A x T x N	1	18.91	.44	18.91	.03
4-way Interactions	5	21.66	.51	792.52	1.28
G x I x A x T	1	12.66	.30	476.10	.77
G x I x A x N	1	26.41	.62	2,560.00	4.14
G x I x T x N	1	.16	.00	166.06	.27
G x A x T x N	1	12.66	.30	354.03	.57
I x A x T x N	1	56.41	1.32	406.41	.66
5-way Interactions	1	7.66	.18	455.63	.74
G x I x A x T x N	1	7.66	.18	455.63	.74
Error	128	42.63		618.53	
Total	159	52.06		679.66	

* Significant at the 1% probability level

All YL media compositions tested in the primary culture stimulated the proliferation of glistering white calli on the cultured seedling internodal sections. The callus tissue consisted of paranchymatous cells (Figure 7). When these calli were subcultured three times, at four week intervals on the primary culture, they continued to grow without initiation of shoots or roots on any of its media compositions. Moving the callus to the first transfer (which contains different types or concentrations of growth regulators as shown in Table 1) did stimulate organization capacity on all of its media compositions. After two to three weeks in the first transfer, the calli produced a large number of white and pale greenish dome-like structures that became visible at several loci of the callus (Figure 8). These structures were characterized by concentrations of dividing, small, and densely cytoplasmic cells and seemed to originate along the periphery of the callus. These gradually became green and organized into shoot apices (Figure 9). Soon leaf primordia arose and gradually a whorl of leaves organized around the apices with the eventual formation of shoots (Figure 8). In many regions of the callus, differentiation of vascular tissues took place either before or along with shoot differentiation (Figure 10). Although most of the shoots appeared normal, some abnormal ones were observed. These abnormalities were manifested mainly by the formation of shoots with narrow leaves. Occasional rooting of these shoots also occurred (Figure 8). During the organization of these roots, meristematic areas were differentiated within the callus (Figure 11). These meristematic pockets then pushed out of the callus, in the immediate vicinity near the base of the shoot, and formed roots (Figure 12).
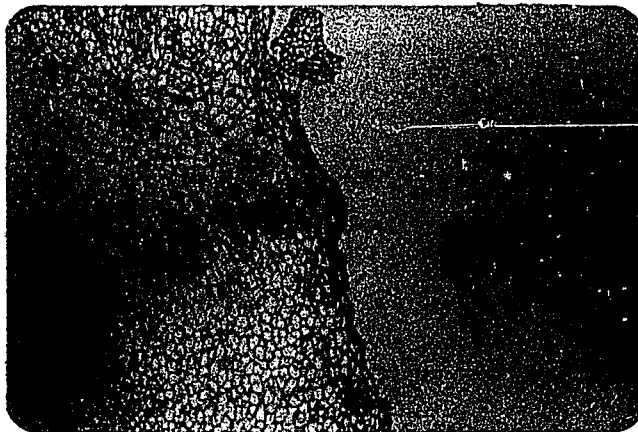
Figure 7. Histological section of papaya callus
showing paranchymatous cells. x 260.

Figure 8. Papaya callus with dome-like structures
that developed into shoots and roots.
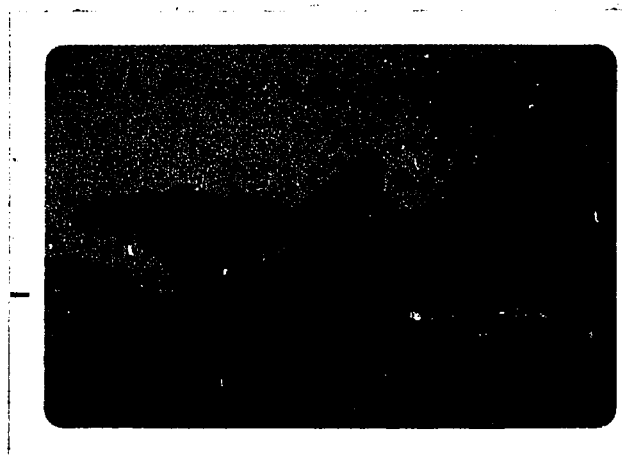
Figure 9.   Histological section of papaya callus showing
            shoot apex regeneration. x 100.
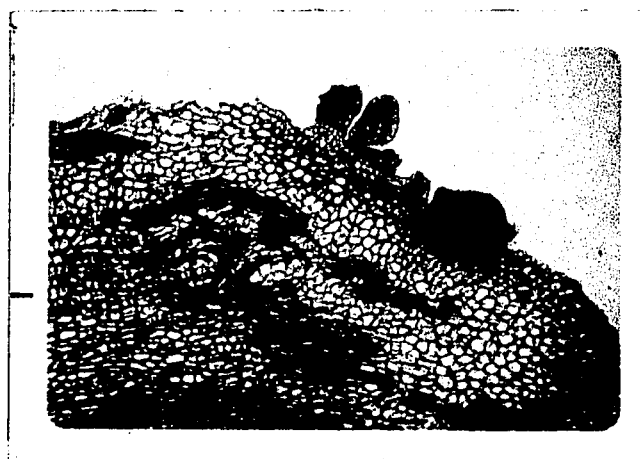

Figure 10. Histological section showing vascular tissue
            formation along with shoot apex regeneration
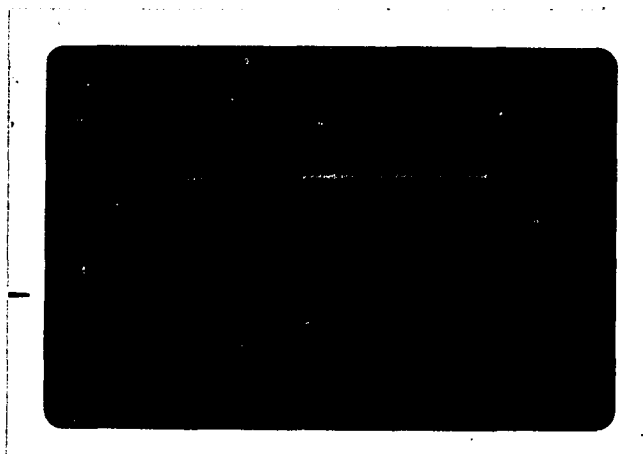   in papaya callus. x 325.

Figure 11. Histological section showing root differ-
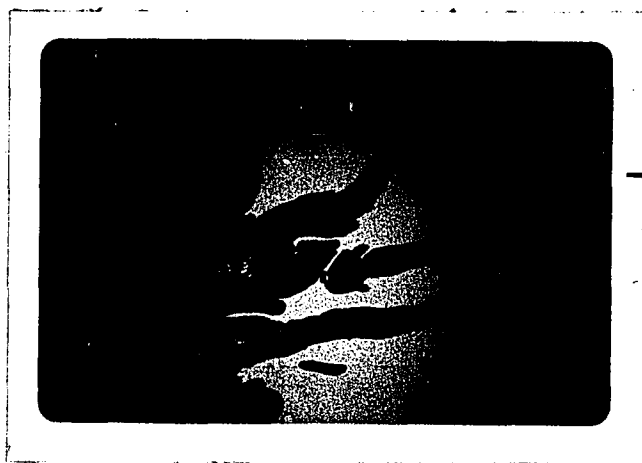entiation in papaya callus. x 1463.

Figure 12. Histological section of papaya callus
showing root formation near the base of
a shoot. x 130.

These roots, however, were thin and lacking in root hairs. Rooting was enhanced, except for the media composition in which glycine, adenine and thiamine were omitted (Table 9), when the shoots were separated from the callus and moved individually to the second transfer.

Some plantlets appeared to develop from embryoids which were often found in surface regions and occurred in the same callus, proliferating from cultured internodal sections, which regenerated shoots. Figures 13 and 14 show these embryoids at the globular and heart stages, respectively. Precocious internal differentiation of vascular strands was also observed in most of these embryoids (Figure 14). Eventually, these embryoids developed further by forming cotyledon-like appendages and shoots with true leaves. There were occasional variations in the development of these embryoids resulting at times in distorted cotyledons, narrow leaves and distorted shoots.

Data of the experiment where pyridoxine and boric acid were omitted singly and in combination showed that the presence of these two substances in the medium did not cause significant effects on the growth value and organogenesis of either cultured shoot tips or internodal sections (Tables 4, 5 and 6).

Establishment of Tissue Culture
Derived Plants in Soil

The tissue culture-derived plants were removed from the culture vessels, washed free of callus and agar, and transplanted to 10 cm styrofoam cups containing an equal volume mixture of perilite and peat-moss. Drainage holes were punched in the base of the cups. The potted plants were placed in the growth room under a light intensity of
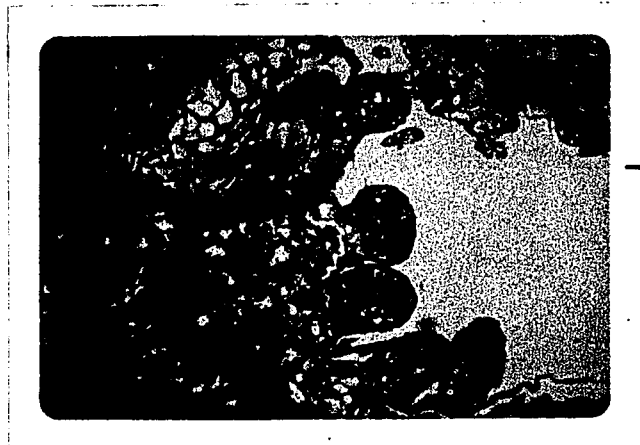
Figure 13. Histological section of papaya callus showing
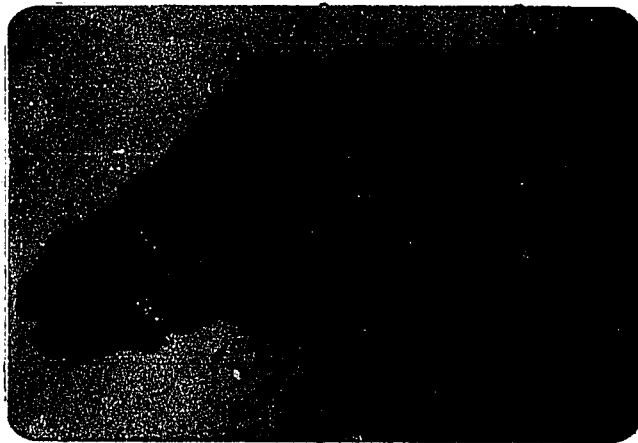embryoids at the globular stage. x 650.

Figure 14. Histological section of papaya embryoid
at the heart stage. x 260.

10,000 lux. Each established plant was covered with an inverted beaker that was removed gradually over a period of two weeks. Plants were watered every other day. They finally were transplanted into 15 cm plastic pots containing the same soil mixture and were placed in a greenhouse under intermittent misting (10 sec/6 min) for two weeks and then watered by hand. Temperature in the greenhouse ranged from approximately $26^{\circ}C$ during the day to $21^{\circ}C$ during the night.

A fertilizer of an analysis of 20-20-20 (Peter Co., Allentown, Pennsylvania) was applied once every week after the plantlets were removed from the culture vessels.

Root hairs were lacking on most plantlets grown on the modified media of MS and YL. This was especially apparent with roots growing into the agar media as roots growing on the surface of the agar medium formed adequate root hairs (Figure 6). Some investigators, working with the in vitro propagation of other plants reported difficulty in establishing these plants once they were removed from the nutrient medium and planted in soil (Pillai and Hildebrandt, 1969). Soil establishment of papaya plantlets which lacked adequate root hairs was not a problem as new roots and root hairs regenerated five days after planting in soil mixture.

All young plants were lost during attempts to transplant them without the acclimatization process. The procedure permitted a survival rate of 85% of the transplanted plants. Figure 15 shows representative plants established under greenhouse conditions.

Figure 15. Tissue culture-derived plants established in soil in a greenhouse.

<u>Date Palm</u>

Disinfection of Plant Material

The procedure applied by Reuveni and Lilien-Kipnis (1974) for disinfecting date-palm embryos was used first. The results of this were cultures with a contamination rate of up to 68%. Consequently, experiments were conducted with several sterilants (household bleach, ethyl alcohol, and amphyl) being tested at different concentrations and times of application. Best results[1] were achieved when the procedure employed by Reuveni and Lilien-Kipnis (1974) was modified. The modifications included the scarification of the seed before their surface sterilization and immersing the excised embryos in 2% amphyl for 1 minute.

For disinfecting male flowers, an experiment was employed during which household bleach, ethyl alcohol, and amphyl were tested at different concentrations and times of applications. External flaming of the unopened spathes and flowers was used both alone and before using the sterilants. Best results were obtained when the male flowers were immersed in 5% household bleach for 5 minutes followed by rinsing three times with sterile distilled water. Higher concentrations and longer immersion periods in household bleach and the use of ethyl alcohol and amphyl in all concentrations and immersion periods caused explants to die after three days in culture. External flaming of the unopened spathes and flowers used both alone and before using the sterilants tested also caused the cultured flowers to die after three days in culture.

[1]See Materials and Methods for detailed disinfection procedures.

Anthers were removed from the surface-sterilized flower buds and cultured without any further sterilization procedure.

Bud Culture

Preliminary work was conducted in July, 1976 in order to test the work technique, environment conditions, and nutrient media for culturing buds located between the last emerged leaf (the spike leaf) and apical meristem of 5 year-old offshoots. The work was performed on two offshoots obtained from the University of Arizona Citrus Experiment Station, Mesa, Arizona. Buds were excised and cultured on the complete MS medium containing 2 g/l PVP according to the procedure employed by Reuveni and Lilien-Kipnis (1974). Agar and stationary liquid cultures were used. Filter-paper bridges were used to support the tissues during the use of liquid cultures. All buds cultured on agar media showed some growth but browned and died after 30 to 50 days. In liquid cultures, the buds dried up after two weeks apparently due to the small area of contact between bud base and support.

The work was then discontinued as the number of offshoots available was insufficient for further experimentation.

Excised Embryo Culture

The effects of the addition of 0 to 10 mg/l each of kinetin and IBA to the MS medium, supplemented with 2 g/l PVP, on the growth and development of excised embryos were studied. The cotyledon, cotyledonary sheath and radicle became distorted when kinetin was added alone at the concentrations 0.001 to 1 mg/l (Figures 16A and C). When 2 mg/l kinetin was added embryo germination was similar to regular seed

Figure 16. Excised date palm embryo germination _in vitro_ as affected
by adding to the MS medium 0 to 10 mg/l each of kinetin
and IBA.

A. 0 to 0.5 mg/l each of IBA and kinetin.
B. 1 to 10 mg/l IBA and 0 to 0.5 kinetin.
C. 0 to 0.5 mg/l IBA and 1 to 10 mg/l kinetin.
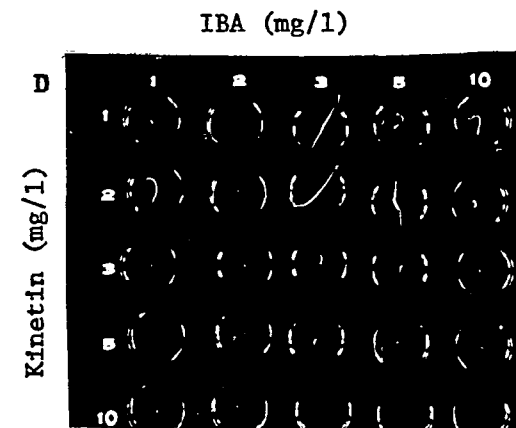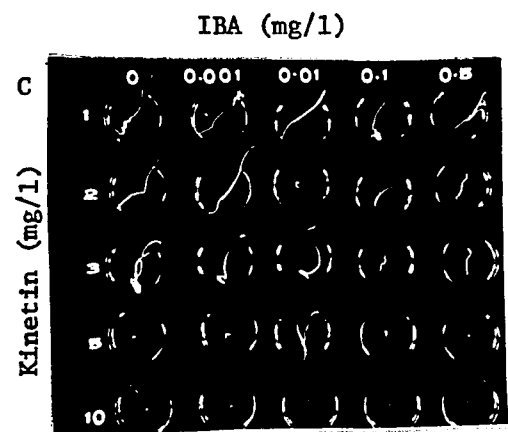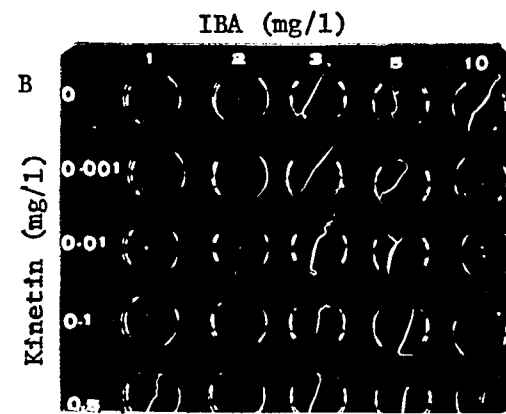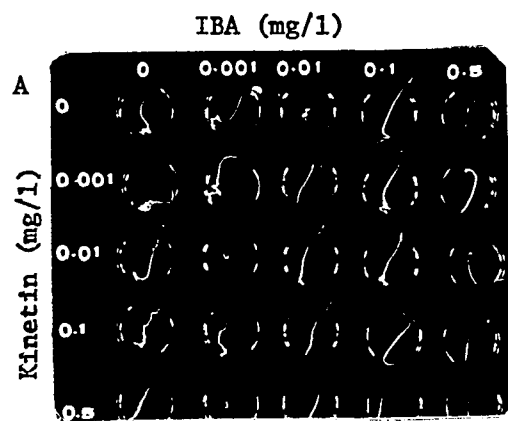D. 1 to 10 mg/l each of IBA and kinetin.

Figure 16. Excised date palm embryo germination *in vitro*.

germination wherein the cotyledonary sheath elongated and the primary root emerged and occasionally one or two leaves developed (Figure 16C). At 3 mg/l, kinetin caused the radicle to become distorted but the cotyledon and the cotyledonary sheath elongated normally with the formation of two leaves (Figure 16C). No embryo germination was obtained when kinetin was added at the concentrations 5 and 10 mg/l. IBA added alone, however, was inconsistent in its effects by causing germination at the concentrations 0 to 0.1 mg/l. It prevented germination at the levels 1 and 2 mg/l, but again stimulated germination at concentrations of 3 to 10 mg/l. When both kinetin and IBA were present in the medium, in combination, the responses of the embryos ranged from normal to no germination (Figure 16 A-D).

Generally, the excised embryo germination in vitro occurred 2 to 3 days after culture. This is compared to seed germination in soil which takes 7 to 10 days.

Small, 7 mm in diameter, green and compact callus developed from the cotyledon when 0.01 mg/l IBA was added alone (Figure 16A). Attempts were made to induce organogenesis on the callus. These attempts were unfruitful as the callus browned and development ceased in two subcultures.

Male Flower and
Anther Cultures

An experiment was first conducted to select the stage of development of the male spathe and the location therein of flowers which would yield a high percentage of pollen at the unicleate and binucleate stages. No study was made of the relationship between the age of the

spathes and the pollen stage of development. Yet the experiment showed that as long as the spathe was located at the base of the palm's head and less than 20 cm long a higher yield of uninucleate and binucleate pollens could be obtained. The experiment also showed that the upper half of the flower strand yielded 100% uninucleate pollens and the lower half produced 75% uninucleate and 25% binucleate pollens (Figure 17). For this reason, only spathes measuring less than 20 cm long and located at the base of the palm's head were used.

Unopened spathes were kept in a cold room at $4^{o}C$ for 48 hours and then opened in a sterile glass-fronted hood where the flowers were removed singly and surface sterilized. Flowers and excised anthers were then cultured on the complete MS medium supplemented with 2 g/l PVP. 0 to 5 mg/l kinetin was added singly and in combination with 0 to 5 mg/l NAA and 0 to 10 mg/l IAA. No organogenesis was observed during 40 days of culture although greening of some of the flowers occurred six days after culture (Figure 18). Therefore, data regarding the number of greening flowers per treatment was collected, and the results are shown in Figures 19 and 20. Figures 19 and 20 show that no greening occurred on the medium containing no growth regulators, and that 1 mg/l kinetin, added alone, gave the highest number of greening flowers. It also can be seen that neither NAA nor IAA, used alone or in combination with kinetin, produced a number of greening flowers greater than that produced when 1 mg/l kinetin was added alone.

The perianth was the only part of the flower that greened. The six attached anthers were all browned (Figure 18). Flower and media browning was the major problem encountered. All cultured flowers

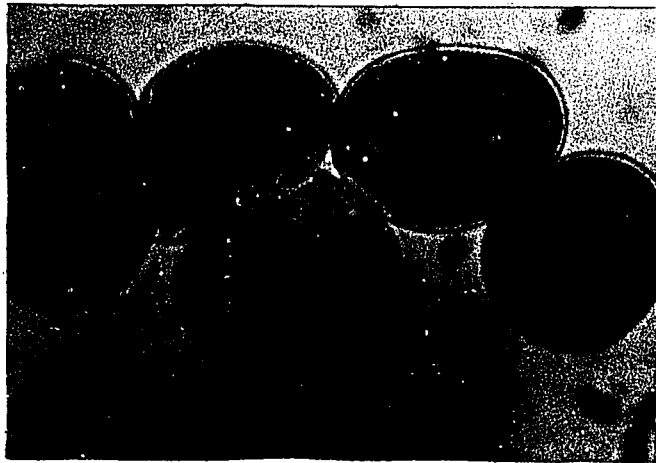Figure 17. Date palm pollens at the uninucleate stage.
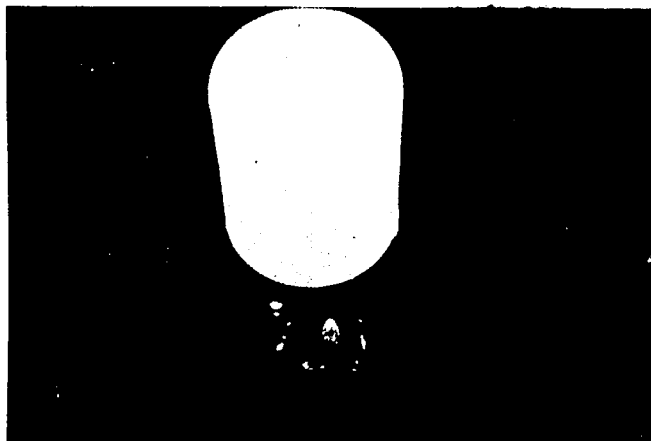x 2750.

Figure 18. Greening of date palm flower in vitro.
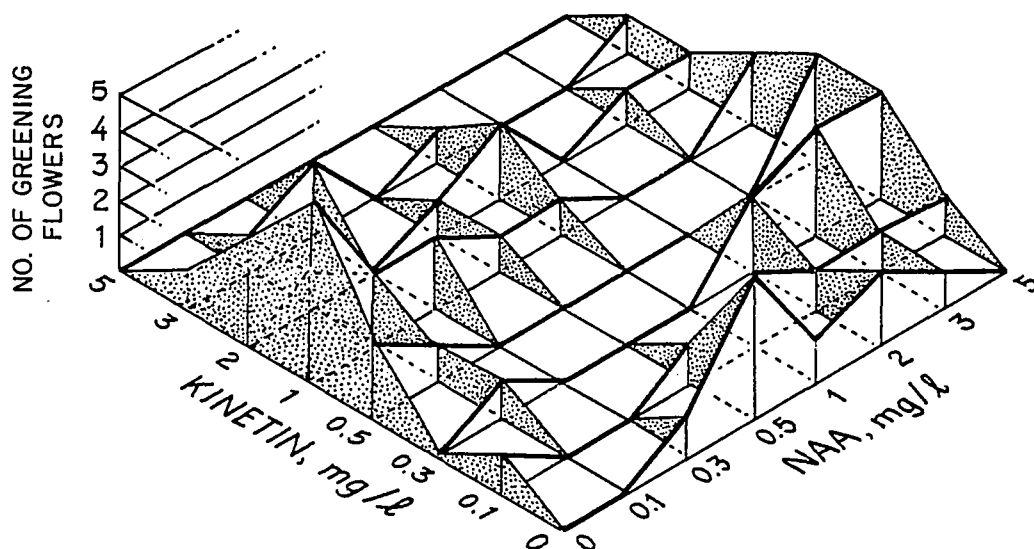
Figure 19. Number of date palm male flowers that greened _in vitro_ as affected by adding to the MS medium 0 to 5 mg/1 each of kinetin and NAA.
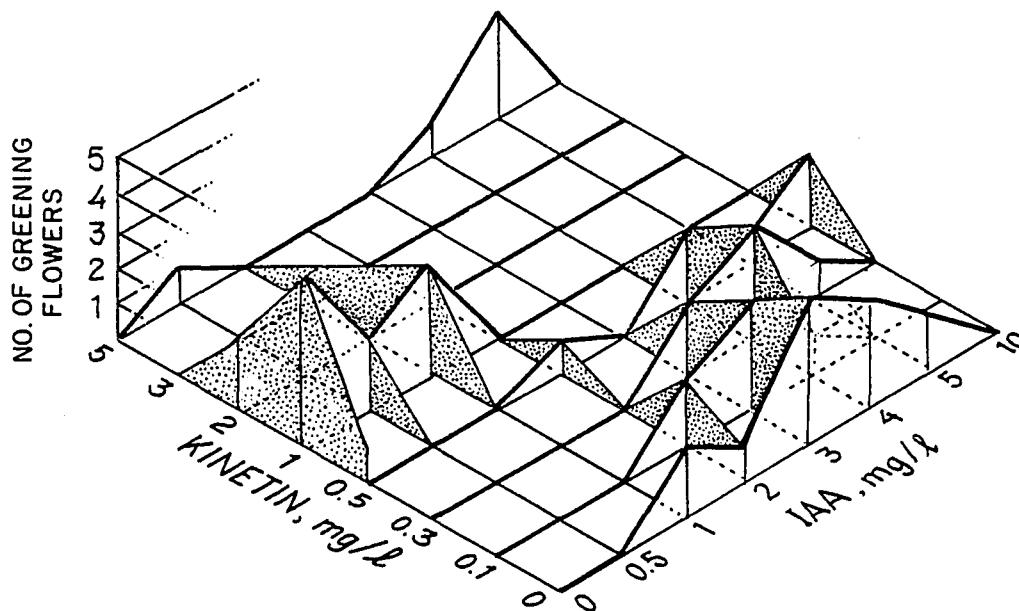
Figure 20. Number of date palm male flowers that greened _in vitro_ as affected by adding 0 to 5 mg/1 kinetin and 0 to 10 mg/1 IAA.

secreted a brown substance from the area where they were originally detached from the flower strand (Figure 18). A substance diffusing from the flowers colored the medium first yellow, then brown and finally black. The substance discharged into the media caused the whole flower to turn black and its eventual deterioration and death.

Anthers, excised from surface sterilized flowers, were also cultured on each type of media used to culture the male flowers. In all cases anthers browned and died after an average of seven days in culture.

DISCUSSION AND CONCLUSIONS

Papaya

In this study, in vitro cultured papaya explants were shown to differentiate into plantlets and that it was unnecessary to use complex media when simpler ones were adequate. The formulaes of both MS and YL media were simplified considerably by eliminating several of their nutrient constituents. The presence of glycine, myo-inositol, nicotinic acid, pyridoxine and boric acid in both MS and YL media as well as casein in MS medium resulted in little or no stimulation to either growth or development. Of the eight substances tested only thiamine and adenine were important to the growth and organogenesis of the cultured explants. Presence of thiamine in both MS and YL media increased the number of rooted shoots. Presence of adenine in YL medium, on the other hand, increased the number of shoots but supressed rooting.

The unimportance of glycine, myo-inositol, nicotinic acid, pyridoxine and casein for papaya explants is consistent with findings of several tissue cultures on other plants. Linsmaier and Skoog (1965) disclosed that glycine, nicotinic acid, and pyridoxine were not required for tobacco growth and development. Murashige and Tucker (1969) also found that glycine, nicotinic acid, pyridoxine, and casein were not required as critical factors for lemon callus growth. Torrey and Reinert (1961) questioned any beneficial effect of myo-inositol in media on which they cultured carrot tissues. Other workers proving the

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unimportance of one or several of the above compounds include:
Hildebrandt, Riker and Duggar (1946), Riker and Gutsche (1948),
Czosnowski (1952), Steinhart, Standifer and Skoog (1961), Risser and
White (1964), Steinhart, Anderson and Skoog (1962), Schenk and
Hildebrandt (1972).

The insignificance of glycine, myo-inositol, nicotinic acid,
and pyridoxine in the tissue culture media may indicate that adequate
synthesis of these compounds occurs in papaya tissues. Casein hydrol-
ysate is a collection of amino acids (LaMotte, 1960). Since casein
was also proved here to be an unnecessary media amendment for papaya
culture, one may also suggest that adequate synthesis of the amino
acids making up this compound occurs in papaya tissues.

The thiamine effect shown in this research was in agreement
with its well known effect in promoting the development of auxin-
induced rooting of cuttings. Linsmaier and Skoog (1965) noted that
thiamine promoted root development on tobacco callus. Murashige et al.
(1972), working with the tissue culture of asparagus, found that
thiamine stimulated rooting and increased vigor of roots but did not
influence shoot formation. Murashige and Tucker (1969) discovered that
thiamine was so essential for lemon callus that almost no growth oc-
curred when it was withheld from the nutrient medium. Other researchers
realizing the importance of this vitamin as an addendum in the plant
tissue culture media include: Bonner and Addicott (1937), White (1943),
Steinhart et al. (1961), White (1963), Witsch (1963).

It seems probable that adenine is a critical factor in pro-
cesses leading to the initiation and growth of buds in many if not all

species of higher plants. Its promotive effect on shoot initiation, and growth was first noted by Skoog and Tsui (1948). Miller and Skoog (1953) found that the number of buds formed on tobacco segments increased markedly by the addition of adenine to the medium. Miller and Skoog (1953) also showed that there was only a slight increase in the number of buds formed on tobacco segments and callus when glycine and nicotinic acid were added to the medium, and that the addition of myo-inositol to the medium did not effect budding at all. Skoog (1950) and Skoog and Tsui (1951) found that adenine promotes budding not only in tobacco stem segments and callus but also in pieces of horseradish roots. Jacquoit (1951) has reported that adenine promotes budding in in vitro cultures of cambial tissues of Ulmus campestris collected during the winter and decreases it in tissues collected during the summer. Vasil and Hildebrandt (1966) found that the addition of adenine to the MS medium stimulated the development of embryoids on parsley callus. Nitsch et al. (1967) demonstrated the promotive effect of adenine on shoot initiation of Plumbago indica internode cultures. Miller and Skoog (1953), working with the tissue culture of tobacco, suggested that presumably adenine also would be effective in many other species if the endogenous auxin levels and deficiencies in other factors necessary for growth did not prevent realization of the response.

Beside adenine, the presence of 15% v/v coconut milk in YL medium seemed to enhance both growth and the number of shoots developed from cultured shoot tips and callus. This was evident from the fact that shoots continued to develop on cultured shoot tips and callus, although in a smaller number, even when adenine was withheld from YL

medium. Furthermore, only one plant was obtained from each shoot tip cultured on the MS medium where neither adenine nor coconut milk was added. It is also worthwhile to mention that the presence of casein hydrolysate in the MS medium did not replace the effectiveness of coconut milk, present in YL medium, in inducing multiple shootlets. Coconut milk has been found as in the case of casein hydrolysate, to contain a collection of amino acids (Tulecke et al., 1961). This suggests that the coconut milk contains some stimuli, not found in casein hydrolysate, which enhance the formation of multiple shootlets on cultured shoot tips and both shoots and embryoids on callus. However, the nature of stimulatory substances contained in coconut milk has not yet been entirely elucidated.

The omission of glycine, myo-inositol, casein, adenine, thiamine, nicotinic acid, pyridoxine, and boric acid, in the concentrations ordinarily included in the MS medium and its modifications was studied. Further tests are necessary to settle the question of whether different levels of these, or of other nutrient supplements which are sometimes used, actually have some effects on growth and development of papaya explants. Substances generally considered as growth factors for higher plants have been reviewed by Aberg (1961) and vitamin mixtures, etc. employed for plant tissue cultures are discussed by Murashige (1974). Conceivably any normal metabolite and any substance capable of replacing or being converted into another might under appropriate conditions promote in vitro growth of some tissue. Therefore, no definite limit can be set to the number of potential growth factors.

The response of papaya explants to kinetin and auxins was in general agreement with results obtained in plant tissue culture. Roots were induced on differentiated shoots when the medium contained more auxin and less kinetin, i.e., when proportions required for shoot formation was reversed.

Some papaya plants appeared to develop from embryoids which were often found in surface regions and occurred in the same callus which regenerated shoots. Occasional formation of embryoids also took place on callus originating from cultured shoot tips. The development of these embryoids may be very similar to that of zygotic embryos and that callus paranchyma cells may, under appropriate nutrient conditions, recover the totipotency that was originally inherited in the egg.

The reproducibility of results from one experiment to the next has been satisfactory, but some variability was observed in total yields and morphogenetic patterns in response to a given treatment. Variability from one replicant to another within a given treatment was high in some cases. Inconsistancy among replicants was more pronounced in growth values of the tissues than in the morphogenetic patterns. Variability from one experiment or replicant to another may be attributed in part to differences in environmental conditions. The reported yields and organogenesis were obtained with the temperature of the growth room kept at $27^{o}C$ but temperature varied occasionally due to power failure, although its influence was not systematically studied. Other possible factors contributing to the inconsistency from one experiment or from one replicant to another include differences in the nutrient status of the plants from which the explants were excised and

differences in callus size and density. Furthermore, growth value of
the tissues was calculated on the basis of fresh weight. Fresh weight,
although non-destructive, is influenced by the water content of the tis-
sues.

In the case of shoots and embryoids embedded in totipotent
callus, one must distinguish between the effect that exogenous growth
substances might have when in direct contact with these organized
structures and the effect when such substances are modified by inter-
vening living tissues. This situation occurs in callus, where shoots
and embryoids are insulated from the medium by varying amounts of callus
parenchyma. The occasional rooting of some of the shoots and embryoids
(Figure 8) before they were transferred to the rooting medium may re-
flect the variety of localized micro-environment that exist within a
single callus body.

In the experiment of culturing shoot tips of 2 year-old plants,
the terminal buds were removed from some plants to stimulate the dor-
mant axillary buds to grow. Plantlets were obtained from both terminal
and lateral tips. This is very important in horticultural practice
because all available tips on the plant can be used with an equal
chance of successful in vitro propagation.

The fact that plantlets were obtained from shoot tips excised
from both seedlings and 2 year-old plants indicates that the ability of
in vitro propagation of papaya plants is independent of age.

Murashige (1974) noted that the successful rooting and transfer
of plantlets from axenic cultures to soil is one of the more difficult
and neglected technical problems in the in vitro propagation researches.

Conditions found to be the optimum for multiplication of plants _in_
vitro may be inadequate to enable successful subsequent transfer and re-
establishment in soil of the plants. For papaya, the conditions of en-
vironment must be immediately suitable once transfer has been made from
culture tubes to greenhouse. Covering the potted plants with inverted
beakers in the growth room under higher light intensities (10,000 lux)
and maintaining them under misting in a greenhouse appeared helpful.
These conditions probably contribute to retarding desiccation. All at-
tempts to establish tissue culture-derived plants in soil without the
acclimatization process resulted in the loss of all plants.

Histological examination during the development of the new
shootlets obtained from cultured shoot tips on modified YL medium re-
vealed that many of them arose from axillary buds. In view of this
pattern of organogenesis it is not surprising that the papaya plants
derived from this method of multiplication maintain the diploid char-
acteristic of the original plants. These shoots were rooted to form
complete and morphogenetically normal plants (Figure 15). Nevertheless,
some abnormal shoots with narrow leaves were observed originating on
some of the totipotent calli. Interestingly enough these shoots did
not root when separated and transferred individually to the rooting
medium. Thus, papaya may be like asparagus (Takatori, Murashige and
Stillman, 1968) and grape (Krul and Worley, 1977) in that plants with
an abnormal genetic composition do not form viable plants. Although
the _in vitro_ propagated papaya plants appeared "normal" it will be
necessary to await cytological analysis and development of the plants
to the fruiting stage to confirm this observation.

The basic parameters in the application of tissue culture method towards an asexual multiplication of <u>Carica papaya</u> L. seem established. These include the refinement of nutrient media, culture conditions, and steps in transplanting. Tissue culture-derived plants were also obtained by using explants excised from full-grown plants instead of seedlings which may provide a better opportunity for the selection and the subsequent clonal propagation of outstanding papaya plants. The feasibility of commercial application of this technique for the asexual propagation of papaya plants remains to be demonstrated. The development and establishment of a commercial plant tissue culture laboratory has been reviewed by Holdgate and Ansley (1977) and the principles for reducing costs for commercial tissue culture propagation have been discussed by Fossard and Bourne (1977).

Date Palm

The objective of this study was to find a new and rapid method for the propagation of the date-palm using tissue culture. Excised embryos, buds, male flowers, and anthers were cultured on the complete MS medium containing 2 g/l PVP and different types and concentrations of growth regulators.

Of the tissues tried, only embryos could be cultured successfully. Small green compact callus developed from the cotyledon when 0.01 mg/l IBA was added alone but the callus died during subculturing.

In spite of greening of the male flower's perianth, the potential for regeneration which apparently exists in these organs of other plants (Crisp and Gray, 1975; Furukawa, Yamada and Kobayashi, 1975;

Anderson et al., 1977) was not expressed in this study of the date

palm. The _in vitro_ greening of flower's perianth may nevertheless in-

augurate an avenue of research which may prove fruitful in the future.

The main problem encountered with all tissue and organs, ex-

cept for excised embryos, was the discharge of a brown substance from

the explants into the medium which caused the eventual deterioriation

and death of these explants. The addition of PVP, a substance known to

prevent browning of the tissue, showed no beneficial effect. The prob-

lem of browning was not specific to the explants only. The callus,

developed on the cotyledon, also browned upon subculturing. Excised

embryos did not brown presumably because intact and uninjured embryos

were used.

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