****

CHEMICAL NUTRITIONAL AND BIOCHEMICAL STUDIES IN PLANT TISSUE CULTURE SUCH AS TECTONA GRANDIS (TEAK).

A
THESIS
SUBMITTED TO
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

For
THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

By

(Miss) BHARATI R. DHRUVA

Division of Biochemistry
National Chemical Laboratory
POONA 8, INDIA.

1974

****
****

CONTENTS

##########

A C K N O W L E D G E M E N T

The author wishes to record her deep sense of gratitude to Dr. V. Jagannathan under whose guidance these investigations were carried out.

The author also gratefully acknowledges the valuable help given to her by her colleagues, Dr. A.V. Rama Rao and Dr. A.F. Mascarenhas. Thanks are also due to Dr. S.B. David for useful discussions.

The author is indebted to the Director, National Chemical Laboratory, and to Dr. K. Venkataraman, Principal-Investigator, PL-480 scheme on Wood Phenolics, for permission to submit this work as a thesis for the degree of Ph.D.

#######

INTRODUCTION

GENERAL INTRODUCTION

Though viable plant tissue cultures of carrot and tobacco were established by Gautheret (1939), Nobecourt (1939) and White (1939) more than thirty years ago, very few woody trees have been grown in vitro as cultures which are viable on prolonged sub-culture. White (1931) outlined the use of plant tissue cultures for studying fundamental problems in plant cytology and physiology. This technique has been successfully used for the propagation of forest trees, to study biogenesis of secondary products, for evaluation of plant growth regulators and for the study of their genetics (Staba 1963). The objective of the work reported in this thesis was to establish tissue cultures of a few forest trees and to study the formation and biosynthesis of phenolics by these cultures. The work consists of the following: (1) establishment of continuous tissue cultures of forest trees and study of their nutritional requirements; (2) isolation and identification of phenolics occurring in the callus cultures and (3) study of the biosynthesis of the phenolics.

History

Haberlandt (1902) was the first to suggest the use of tissue culture as a possible tool to study the interrelations and reciprocal influences to which cells are subjected in a multicellular organism. His attempt to cultivate plant cells in vitro was a failure due to a lack of knowledge of plant nutrients and the use of highly differentiated cells. Though Kotte (1922a, 1922b) and Robbins(1922a, 1922b) independently were successful in growing for a short period

roots of certain species of grass on media containing glucose and inorganic salts, the cultures failed to survive on sub-culture. White (1934) was the first to establish a continuous culture of root tips of tomato on media consisting of inorganic salts, yeast extract and sucrose. This was an organ culture having normal morphology and anatomy. Tissue cultures of undifferentiated callus capable of growing indefinitely in vitro were established by Gautheret (1939), White (1939) and Nobecourt (1939) using carrot and tomato. After these pioneering studies, this technique has advanced rapidly and many viable callus cultures have been established, which have been used for the study of a wide range of problems related to plant biochemistry, physiology and agriculture.

SECTION 1

Cultivation of callus tissue of trees in vitro

Callus cultures have been a major type of tissue culture used for many basic studies. Tissues have been isolated and established from many species and parts of plants. The undifferentiated cell masses have been established from embryos (Carew and Schwarting (1959), excised roots (Torrey and Skigemura, 1957), coniferous seedlings (Loewenberg and Skoog, 1952), redwood burls (Ball 1955), crown gall tumors (Hildebrandt and Riker, 1949, 1959), virus induced and insect induced tumors (Pelet, Hidebrandt, and Riker and Skoog, 1960), fleshy roots like potato (Steward and Caplin, 1951) or carrot (Black, 1958) (Nobecourt, 1939) and stem tissue(White 1939).

Angiosperm tree species were employed even during the early development of the technique of plant tissue culture, and tissues of a few hardwood tree species have since been maintained in culture. In his book Gautheret (1959) lists 20 angiosperm and 12 gymnosperm tree species cultured *in vitro*, of which only 4 angiosperm tree species were viable on prolonged sub-culture whereas the rest were short term cultures.

The modification in the composition of salt solutions and isolation and identification of various plant growth regulators has made it possible to establish continuous culture of different tissues tree species. On the following pages a brief review of the trees cultivated *in vitro* is given (Table 1) .

Table - 1

Trees cultivated in vitro

Angiosperm

No.	Species	Nature of tissue	Media	Remarks	Reference
1	2	3	4	5	6
1	Asimino triloba	N	Mineral salts + leaf extract	-	Gautheret (1959)
2	Acer pennsylvanicum	N	High salt media + NAA + CM 25 %	+	Mathes (1967)
3	A. pseudoplatanus	N	Heller's mineral + thiamine + pantothenate, 2,4D + 10% CM	+	Henshaw et al.(1966)
4	A. saccharum	N	High salt med.+NAA + CM 25%	+	Mathes (1967)
5	Betula verrucosa	N	Mineral salts + vit.	+	Jacquiot (1964)
6	Castnea vesca	N	"	-	Jacquiot (1950)
7	Chiplosis lineris	T	"	+	Gautheret (1959)
8	Citrus limonum	N	Mineral salts + NAA, Morel's vit.+15% CM	-	"
9	C. limonum	N	Mineral soln. + NAA, Vit. B_1	+	Gautheret (1959)
10	C. aurantium	Fruit peel	Murashige & Skoog's supplements, vitamins at higher conc. + CM + CM 20%	+	Brunet & Ibrahim (1973)
11	C. medica	"		+	"
12	Crataegus monogyna	N	Mineral salts, NAA + Morel's vitamin	+	Gautheret (1959)
13	Fraxinus excelsior	N	Mineral solution	-	Jacquiot (1964)
14	F. pennsylvanicum	N	Modified Reinert & White, 2,4D, myo-inositol, pyridoxine + kinetin	-	Wotert and Skoog (1966)
15	Hevea brasiliensis	N	Mineral salts + auxins + vit + amino acids	+	Chau (1969)

Table 1 contd.

1	2	3	4	5	6
16	Mangifera indica	leaf T	Whites minerals + 3 g./l CH	+	Jain & Arya (1964)
17	Persea americana	Coty-ledon	Mineral soln. + 2,4D + 6-10-7 + 18% CM	-	Gautheret (1959)
18	"	N	" +2,4D 10^{-7} + 15% CM	-	"
19	Populus deltoides	N	Mineral salts + NAA + 2,4D + Vit.B_1 + panto-thenate + glycine + 15% CM	-	"
20	P. tremula	N	"	+	Jacquiot (1964)
21	P. tremuloides (Triploid)		Mineral salts + 10% CM	+	Mathes (1964)
22	Prunus ariom	N	"	+	Jacquiot (1964)
23	Pyrus communis	N	" NAA	+	Gautheret(1959)
24	Quercus alba	N	Mineral salts + NAA + 2,4D+Vit.B_1+pantothenate	-	Gautheret (1959)
25	Q. palustris	N	glycine + CM 15%		
25	Q. palustris	N	Reinert & Whites + Vit. + auxins + kinetins	+	Wolter (1964)
26	Robinia pseudac-acia	N	Mineral salts + IAA + Jacquiot's Vitamins+CM	-	Gautheret (1959)
27	Rosa wichuraiana	N	Mineral salts + thiamine	-	"
28	Salix babylonica	T	" + NAA + thiamine + biotin + pantothenic acid + 15% CM	-	"
29	Salix caprea	N	" + NAA + thiamine + biotin + pantothenic acid	-	"
30	S. nigra	N	" + NAA + Pantothenate + thiamine + glycine+15% CM	-	"
31	Santalum album	N	" + 2,4D + kinetin + 25% YE	+	Rao (1965)

Table 1 contd.

1	2	3	4	5	6
32	Syringa vulgaris	N	Mineral salts + NAA	+	Gautheret (1959)
33	""		" + IAA + inositol + pantothenate	+	"
34	Theobroma cacao	N	Mineral soln. + 7% CM	+	Archibald (1954)
35	Tilia parvifolia	N	Mineral soln. + auxin + vitamins + amino acid	+	Jacquiot (1964)
36	Ulmus americana	N&T	Mineral soln. + NAA + thiamine + pantothenate + glycine + CM 15%	-	Gautheret (1959)
37	U. campestris	N	Mineral solution + NAA + vitamin solution of Jacquiot	-	Gautheret (1959)
			Gymnosperm		
1	Cupressus funebris	N	Mineral salts & 20% CM	-	Gautheret (1959)
2	C. lusitanica	N	High salt medium; 2,4D; 25% CM	+	Borchert (1967)
3	Ginkgo biloba	Pollen	Modified Whites Mineral + 20% CM + 25% YE	+	Tulecke (1953)
4	"	"	Whites mineral + arginine	+	Tulecke (1953)
5	"	Embryo	Mineral salts + 400 PPM CH		Wang & Li (1966)
6	Juniperus communis	N	" + NAA + 15% CM	-	Gautheret (1959)
7	"	N	" + 2,4D + Gonstabel's vitamin soln. + 10% CM	-	"
8	J. virginia	T	Mineral solution + NAA + 2,4D + Vit. B_1 + pantothenate + 15% CM	-	"
9	Libocedrus decurrens	N	Mineral solution + 2,4D + 20% CM	-	"
10	Picea abies	N	Mineral solution, NAA + Vit.+ B_1, choline + 10% ME		"

1	2	3	4	5	6
11	P. glauca	N	White's mineral + IAA 10^{-8} g/ml, Vit.C 10^{-5} g/ml	+	Gautheret (1959)
12	"	T	" + IAA 10^{-5} g/l + Vit.C 10^{-7} g/ml	+	"
13	Pinus banksina	N	Mineral soln. + NAA + Vit. B_1 + choline + hypoxanthine	-	Lowenberg & Skoog (1952)
14	P. clausa	root	"		Barnes Ph.D. dissertation
15	P. monticola	N	"		Harvey (1967)
16	P. nigra	N	Mineral salts + 2,4D + NAA		Bodgavonic (1968b)
17	P. pinaster	N	Murashige & Skoog's mineral + 2,4D, vitamins, kinetin asparagine		Alain (1971)
18	P. rigida	root	Mineral salts + 2,4D + NAA		Barnes & Naylor(1959)
19	P. serotina	"	"		" (1958)
20	P. strobus	N	Mineral salts + NAA + Vit. B_1 + choline+hypoxanthine		Lowenberg & Skoog (1952)
21	P. sylvestris	N	Mineral salts + IAA		Bodgavonic(1968a)
22	Sequoia sempervirens	N	Mineral soln. + IAA	+	Ball (1950)
23	"	N&T	"	-	Gautheret (1959)
24	Thuja orientalis	Pollen	Whites' mineral + 10% CM + 300 ppm.	+	Rao & Mehta(1969)

N = Normal tissue T = Tumour tissue.

ANGIOSPERMS

1. Annonaceae

Lampton in 1953 cultivated Asimina triloba on a medium containing mineral salts and leaf extract. The callus did not survive on prolonged sub-culture.

2. Aceraceae

Cambial tissue of Acer pseudoplatanus was grown (Henshaw, Jha, Mehta, Shakeshaft and Street, 1966) on a medium containing Heller's inorganic salt solution, 2% sucrose, 1 ppm thiamine, 1 ppm calcium pantothenate, 5 ppm 2,4D and 10% coconut milk. 2,4-D During the period of high cell division rate the mean cell size of A. pseudoplatanus cell suspension culture reached its minimum and the average number of cells per cell aggregate its maximum value. Cell separation depended on cell expansion and it did not take place till cell division had stopped. Street et al. (1968) have shown that a high level of kinetin (2.5 ppm) shortened the lag phase and the phase of rapid cell division. GA_3 at an appropriate concentration prolonged the period of rapid cell division and 2,4D was essential for growth but inhibitory for cell expansion at high concentration. Givan and Collins (1967) suggested that in cell suspension cultures of maple, the respiration rate is more closely related to protein content than to change in cell number, dry weight or packed cell volume. Simpkins and Street (1970) have studied the effect of kinetin on carbohydrate and nitrogen metabolism of this culture. Simpkins, Collin and Street (1970) found that cell

suspension cultures of maple can utilize glucose, fructose, galactose or soluble starch. Cell yield increased with sucrose concentration. Nitrate was essential for growth and urea with nitrate enhanced growth. Further enhancement was obtained by the addition of casein hydrolysate or a mixture of amino acids. 2,4D was essential for growth in the absence of or a low level (0.25 ppm) of kinetin. IAA could not replace 2,4D while NAA at 10 ppm permitted a low level of growth with abnormal aggregation. Kinetin alone at 10 ppm promoted a high level of growth, but the culture became brown and showed increasing aggregation.

Mathes (1967) isolated callus cultures of A. pennsylvanicum (moose wood) and A. saccharum (sugar maple) from one year old twigs or from the primary roots of young seedlings on White's major salts, Nitsch's minor elements, 2% sucrose, 10% coconut milk, 0.5 ppm NAA and 0.8% agar. For subculture the medium was changed to double strength, White's major and Nitsch's minor salts, 25% coconut milk, 2 ppm NAA, and 2% sucrose and 10 ppm vitamin C. The root callus of sugar mapple (A. saccharum) was friable and light yellow in colour, while the stem callus was solid and white or brown. The brown coloration was reduced by the addition of vitamin C and the callus had the ability to utilize soluble starch, galactose and cellobiose as carbon source (Mathes, Morselli and Marvin, 1971). The moose wood stem callus was light tan in colour and friable. This callus could utilise sucrose, glucose and raffinose most efficiently and cellobiose, galactose and maltose to a lesser extent (Mathes 1967).

3. Anacardiaceae

Leaf gall tissue of Mangifera indica was cultivated in vitro by Jain and Arya (1966). They found that White's basal mineral salts agar medium when supplemented with casein hydrolysate gave the best growth. Pancreatic digest of casein at 5g/l gave highest yield of tissue. At pH 5 the tissue grew well without brown pigmentation. Maltose and sucrose at 2.5% gave maximum growth. The tissue showed signs of dryness and excessive friability after two weeks of growth at a concentration below 1.5% of carbohydrate.

4. Bignoniaceae

Tumor tissue of Chilopsis linearis was cultivated by Morel (Gautheret 1959) on a mineral salt medium without any growth promoting substances. The callus was viable on sub-culture.

5. Euphorbiaceae

Chau (1966) cultured Hevea brasilliensis in a medium containing diverse minerals, amino acids, vitamins and growth regulators. It was observed that sucrose was the best source of carbon and a pH below 6 was essential. The callus tissue produced roots if incubated for a long period.

6. Fagaceae

Castanea vesca stem callus was isolated by Jacquiot (1959) on Knop's half strength salt solution with various vitamins, amino acids and other growth factors. The survival on sub-culture was not reported.

Quercus palustris (pin oak) trunk callus culture was established by Wolter (1964) on Reinert and White's medium. The details regarding its nutritional requirements have not been reported.

Quercus alba callus tissue was cultured by Hilderbrandt and Riker (Gautheret, 1959) on a medium containing salts, NAA (10^{-7}M), 2,4D (10^{-6}M), Vit B_1 (10^{-7}M), pantothenate ($3x10^{-6}$M) glycine ($2.5x10^{-7}$M) and 15% coconut milk. The callus died after a few sub-cultures.

7. Lauraceae

Nickell and Morel (Gautheret 1959) tried to establish callus cultures from Persea americana. Nickell's medium contained higher concentration of 2,4D ($6X10^{-7}$M) and CM (18%) than Morel's medium which contained 2,4D (10^{-8}M) and CM (15%). Neither of the callus cultures survived on prolonged sub-culture.

8. Leguminosae

Robina pseudocaria was cultured by Jacquiot (Gautheret 1959) on a mineral salt medium supplemented with IAA (10^{-6}M) and vitamins solution. It was observed that callus grew very well when supplied with coconut milk. Its survival on sub-culture is not reported.

9. Oleaceae

Hildebrandt and Riker (Gautheret 1959) first tried to establish continuous cultures of Fraxinus pennsylvanica on a medium containing mineral salts, α-NAA (10^{-7}M), 2,4D ($6x10^{-6}$M), thiamine (10^{-7} M), pantothenate (2.5×10^{-6}M), glycine ($3x10^{-6}$M) and 15% coconut milk. The callus obtained could not be sub-cultured. Wolter and Skoog (1966) established a viable callus culture of F. pennsylvanica on modified Reinert and White's medium supplementd

with myo-inositol (10 ppm), pyridoxine (0.1 ppm), 2,4D (0.04 ppm), kinetin (1 ppm) and sucrose 2%. The callus had absolute requirements for myo-inositol, pyridoxine and an auxin. The callus could use NAA instead of 2,4D. Kinetin and gibberellin improved the yield while thiamine had no effect.

Jacquiot (1959) cultivated cambial tissue of F. excelsior on Knop's half strength solution, vitamins and growth factors. This callus culture grew at decreasing rates through nine transfers and ultimately died.

Olive callus culture was obtained from young seedlings by Lavee and Messer (1969) on a double strength inorganic modified White's medium. For the early development of callus auxin was not obligatory but after a number of sub-cultures auxin and cytokinin were needed for growth. IAA and 2,4D were most effective for growth. Very short periods of red light illumination were sufficient to effect greening of the tissue. A particularly high ratio of chlorophyll b/a was found under all light conditions and growth was reduced after four sub-cultures in light.

Morel (1948) and Barnoud (1955) separately cultivated tissue of Syringa vulgaris. Morel's callus was obtained on a salts medium with NAA and could grow indefinitely. Barnoud's callus culture was grown on a mineral salts medium having IAA, inositol and pantothenate. The tissue grew in two layers of which one would grow without stimulatory substances (Gautheret 1954)

10. Rutaceae

A short term callus culture of Citrus limonium hypocotyl was obtained by Bove and Morel (1957) on a medium containing a mineral

solution with NAA (5×10^{-7}M) vitamins and coconut milk. Demetriades (1959) established a continuous culture of C. limonium on a medium containing salts, NAA (10^{-7}M) and thiamine (10^{-6}M). (Gautheret 1959).

Citrus medica (Schroeder and Spector 1957) fruit tissues were cultured on modified Nitsch's medium in which yeast extract was replaced by thiamine, pyridoxine, nicotinic acid and glycine. Schroeder and Spector (1957) observed that GA_3 and IAA stimulated callus formation in excised citron tissue and that the tissue responded to a wider concentration range of GA_3 when grown in the presence of IAA.

Brunet and Ibrahim (1973) recently reported tissue culture of Citrus aurantium (fresh orange) and C. medica var linonium (lemon) peel. The callus originated from the flavedo side of the explant on Murashige and Skoog's medium containing a high level of sucrose, B-vitamins, IAA and kinetin. Coconut water was required for the initiation of callus tissue and its continued growth. Orange juice supplied at 10% concentration did not enhance the growth of either lemon or orange callus, which is in contrast to previous report of Murashige and Tucker (1969).

11. Rosaceae

Crateagus monogyna callus culture was established by Morel (1948) on mineral salts medium having α-NAA and vitamins. It was not certain whether the callus required pantothenate. The callus died after a few subcultures. A viable callus culture of Pyrus communis was established by Morel (1948) on a medium containing mineral salts

and NAA. Rosa wichuraiana was cultivated in vitro by Nobecourt and Kofer (1945) on Knop's diluted solution, 2% glucose and IAA (10^{-6} to 10^{-7}M). Thiamine gave the best growth of callus at 2×10^{-6}M. Jacquiot (1959) obtained viable cambial callus of Prunus aviaum on Knop's half strength medium with sugar, vitamins and other growth factors.

12. Saliciae

Gautheret (1959) in 1948 established a short term culture of Salix caprea. The medium for callus growth contained in addition to mineral salts, NAA, thiamine, pantothenic acid, biotin and coconut milk. The callus died after prolonged sub-culture. S. babylonica (Gautheret 1959) crown gall tumor culture established by Bitancourt on mineral salts and 15% coconut milk and S. nigra (Gautheret 1959) cultures on mineral salts together with NAA, thiamine, pantothenate, glycine and 15% coconut milk died after a few sub-cultures.

Populus deltoides callus culture established by Hildebrandt and Riker (Gautheret 1959) on a mineral salt medium supplemented with α-NAA, 2,4D thiamine, pantothenate, glycine and 15% coconut milk was a short term culture. Jacquiot (1959) established callus culture of P. tremula on a half strength Knop's solution with various vitamins and growth factors. On modified Reinert and White's medium, Wolter got viable callus of P. tremuloides (1964). Wolter (1968) found that 2,4D at all concentrations inhibited root formation in P. tremuloides while benzylaminopurine in the absence of auxin initiated shoot formation at certain optimum concentrations, beyond which it was inhibitory. NAA in the absence of cytokinin favoured root formation.

Mathes (1964a) devised a satisfactory medium containing major and trace elements, glycine, thiamine, NAA, sucrose, coconut milk, ferric citrate and agar for callus tissue from triploid P. tremuloides. He observed that 0.05% citrate initiated root formation and 1 ppm IAA together with 0.8 ppm kinetin produced occasional leafy shoots which failed to elongate (1964b). Winton (1968) grew suspension cultures of triploid aspen and observed two kinds of growth, tan coloured smooth and firm spheres. The former grew rapidly at a high concentration of 2,4D (0.5 ppm) the latter grew slowly at a low level of 2,4D (0.04 ppm) and kinetin. With low level of 2,4D alone rooting increased with the age of the source of tissue rather than initial explant size. Winton (1970) has also reported shoot and tree development from callus tissue of triploid aspen in the presence of BAP (0.15 ppm).

13. Sterculiaceae

Archibald (1954) cultivated isolates of bark of Theobroma cacao in a simple Gautheret's medium and observed the formation of cambial tissue in 2-3 months. Gautheret's medium was found to be better than White's medium. For proliferation of callus, White's medium supplemented with pantothenate and biotin was not as good as pure coconut milk medium. The morphology of callus changed with the medium. With White's medium containing pantothenate and biotin it was compact white and granular but it changed when sub-cultured on a coconut milk medium. It also showed a difference when grown on agar slopes and liquid coconut milk medium. On the former it was disorganised, loosely granular, pale brown in colour and translucent, while on the latter it was raised from the surface of the filter paper

by one or two masses of newly formed tissue which was pale brown, translucent, compact but extremely soft and 'mushy'. A smear of the soft tissue across the filter paper support gave rise to masses of the tissue within a month showing that all the cells of the soft tissue are capable of division. Prolonged sub-culture on CM gave rise to colourless tissue having a granular, solid nature.

14. Santalaceae
Sandal-wood

Rao (1965) cultivated Santalum album seeds on a basal medium supplemented with 400 ppm casein hydrolysate and 20% CM. The seeds germinated and produced normal seedlings. Seeds when cultivated on a basal medium containing 0.25% yeast extract, 5 ppm kinetin and 2 ppm 2,4D proliferated into callus tissue capable of unlimited growth and the development of embryos was inhibited.

15. Tiliaceae

Tilia parvifolia cambial tissues were cultivated by Jacquiot (1959) on a medium containing half strength Knop's solution with vitamins and growth factors. Its survival on sub-culture was not reported.

16. Ulmaceae

Normal and tumor tissues of Ulmus americana were cultivated by Hildebrandt and Riker (Gautheret 1959) on a salts medium supplemented with α-NAA, vitamin B_1, pantothenate, glycine and coconut milk. In both the cases of normal and tumor tissues, the concentration of added growth factors was the same except that of coconut milk. Both the callus cultures failed to survive on prolonged sub-culture. Short term callus cultures of U. campestris (Gautheret 1959) were established by Jacquiot on a medium containing salts, NAA, CM and vitamins.

Gymnosperm

There are four gymnosperm families from which 21 different species have been cultured *in vitro*. Only seven of these were long term cultures while the others were short term cultures.

1. Cupressaceae

Two species of the genera Cupressus have been established *in vitro* (1) *C. funebris* and (2) *C. lusitanica*.

A tissue culture of *C. funebris* staminate cone was first derived by La-Rue (Gautheret 1959). The tissue had an essential requirement for coconut milk which was replaced by gibberellin (1 ppm) and casein hydrolysate (2 ppm) in 1960 by Straus and Epp. This is one of the few cultures which requires gibberellin to attain maximum growth. *C. lusitanica* cultures were isolated by Borchert (1967) from the stem of the tree. The explants grew more vigorously on a high salts medium than on Heller's medium and the growth habit changed from compact to friable. Callus could be transplanted to, and maintained on a high salts medium enriched by vitamins but could not survive on Heller's basal medium with or without growth factors. In a high salts medium the addition of 2,4D stimulated proliferation of initial explants and transplanted callus only slightly, while added coconut water was more effective than on the basal medium.

Short term cultures of two Juniperus species were reported. *J. communis* was cultivated by Morel and also by Constabel (Gautheret, 1959). Morel used NAA (10^{-6}M) and CM (20%) with salts while Constabel used 2,4D (10^{-8}M), a vitamin solution and 10% CM. Gall tissue of

J. virginiana was cultivated by Hildebrandt and Riker (Gautheret 1959). The medium was supplemented with NAA (10^{-7}M), 2,4D ($6x10^{-6}$M), thiamine (10^{-7}) and pantothenate ($2.5x10^{-6}$), glycine ($3x10^{-6}$M) and CM 15%.

Straus (Gautheret 1959) established a callus culture from the male cone of Libocedrus decurrens on salt solution supplemented with 2,4D and 20% coconut milk. The culture failed to survive after a few subcultures.

A viable callus culture of pollen of Thuja orientalis (Rao and Mehta 1969) was obtained on Heller's medium with White's micro-elements. The callus was maintained on White's medium supplemented with casein hydrolysate, coconut milk and 2,4D. The cells were uninucleate and filled with starch grains.

2. Ginkgoaceae

Tulecke (1953) was the first to cultivate Ginkgo pollen on White's modified medium containing coconut milk and yeast extract. Coconut milk or aqueous extract of the pollen induced tissue formation. The tissue grew as a mass of undifferentiated cells having a haploid chromosome complement. Morel (Gautheret 1959) established a short term culture of G. biloba on a salts medium supplemented with NAA ($5x10^{-7}$M) and casein hydrolysate. An arginine requiring strain of callus from the pollen was obtained and grown on a medium containing arginine (Tulecke 1960). Yih, Hille and Clark (1966) found that boron deficiency in the medium led to reduction or cessation of growth. Wang and Li (1966) observed that a high (400 ppm or more) concentration of casein hydrolysate induced callus formation in excised Ginkgo embryos.

3. Pinaceae

Two genera have been cultivated in vitro, Picea and Pinus.

Picea abies was first cultivated by Loewenberg and Skoog (Gautheret 1959). The callus has an absolute requirement for malt extract which could be replaced by casein hydrolysate. Arginine, glutamine and urea also supported continuous growth (Steinhart, Skoog and Standifer (1961). Steinhart et al. (1961) have developed a synthetic medium containing inorganic nutrients, NAA, kinetin, arginine or urea, thiamine, choline, cysteine and myo-inositol. They have shown that myo-inositol is an absolute requirement for the growth of the callus.

Picea glauca callus culture was obtained by Reinert and White (1956) on a White's basal medium supplemented with 2,4D, 18 amino acids and 10 vitamins. Tumor tissue of P. glauca had a requirement for IAA (10^{-5} g/ml), vit B_1 and vit C (10^{-5} g/ml). The requirement for IAA was unusual, as most of the genetic and crown gall tumors do not require it. Moreover, requirement for vitamin C was also higher than for the normal tissue (10^{-7} g/ml) (Reinert and Schraudolf 1959). Risser and White (1964) have studied organic growth factor requirements for a strain of cells derived from a tumor of P. glauca. This has resulted in a simpler medium containing White's nutrients, 5% sucrose, 0.25% glutamine, 0.1% inositol and trace amounts of thiamine, niacine, ascorbic acid and 2,4D. Five fold increase in weight was observed in two weeks. White and Gilbey (1966) found that glutamine was essential for P. glauca tissue and any organic

substance or group of substances could not fully replace it. Although ammonium ion was effectively utilized by spruce culture it promoted earlier differentiation in the form of deposition of starch, lignins, tannins, waxy substances and resins, and in one case of reticulated wall thickening. These are all processes characteristic of ageing which are found in maturing regions. While growth with glutamine resembles that of the growing region, White and Gilbey (1966) suggested that glutamine might be an important factor in plant morphogenesis.

Pinus banksiana and P. strobus short term callus cultures were established by Loewenberg and Skoog (1952) on a medium containing malt extract or pine seed extract. The other growth factors were NAA (10^{-6} and 10^{-7}), vit B_1 ($2x10^{-7}$), choline ($5x10^{-7}$) and hypoxanthine (2.5×10^{-7}). Barnes cultivated callus from isolated roots of P. rigida and P. clausa (Barnes and Naylor 1958) and used it for preliminary metabolic studies. P. serotina (Barnes and Naylor 1959) root callus was used to study the ornithine cycle in comparison with excised roots. Bedgavonic (1968a, 1968b) established stem callus cultures of P. sylvestris and P. nigra. P. sylvestris formed callus when apical ends of segments were placed touching the medium which contained no auxin (Bedgavonic, 1968a). On a medium with IAA the rate of callus formation was slowed down when apical ends were placed away from the medium. Also callus formation was more in summer than in winter, and the rate of callus formation was proportionate to IAA concentration. 2,4D (10^{-7}M) favoured callus formation in P. nigra and NAA was found to be useful for obtaining a continuous culture (Bedgavonic , 1968b). Lavaud (1970) observed that on explants

of P. pinaster consisting of phloem-cambium-xylem or phloem-cambium, callus formation was stimulated by IAA, NAA or mixture of NAA and myo-inositol. In the case of a phloem/cambium explant a phloem ligneous generating layer became differentiated between the pre-existing phloem and newly formed parenchymatous tissue. On Murashige and Skoog's salt solution containing 2,4D, kinetin, vitamins and asparagine P. pinaster (Alain 1971) trunk callus culture was established.

Axenic cultures of P. monticula for the study of host-parasite relation between white pine and Cronastium ribicola were established by Harvey (1967). The callus was maintained on a medium containing inorganic salts, NAA, IAA or 2,4D. Amino acids, B-group vitamins and kinetin increased the growth, but were not essential. Cultures with 2,4D did not lose vigor with time when subcultured every month for 12 months. On the other hand cultures with NAA and IAA slowed after 6-12 weeks and could be maintained only for 6 months. The callus was firm but friable and had a well developed chlorophyllous pigment system. The cells were large with well developed nuclei. The cultured cells did not grow on White's or Heller's medium.

P. nigra (Thomas 1972) hypocotyl fragments were grown on Halperin's mineral medium with added sucrose, amino acids, NAA and kinetin. The medium had a high salt concentration and higher concentrations of kinetin and NAA compared to those usually employed in angiosperm cultures.

4. Taxodiaceae

Sequoia sempervirens was successfully cultivated in vitro by Ball (1950) in a simple medium containing Knop's solution, IAA

and sucrose. The callus was able to utilise galactose and mannose as a carbon source. Normal and tumor tissue of S. sempervirens were also grown by Morel (Gautheret 1959) on Heller's medium. Ball (1955) has shown that S. sempervirens can utilize galactose as carbon source.

SECTION 2

The Secondary Plant Products from Tissue Culture

Nickell (1971) defined secondary plant products as "compounds not produced by all plants, whose functions are not known and which are not essential metabolites, but which generally have considerable biological activity".

Among the products identified in plant cell cultures are alkaloids, antibiotics, flavanoids, glycosides, organic acids, phenolics, pigments, saponins, steroids, tannins and terpenoids (Krikorian and Steward 1969).

The isolated products are not always the same quantitatively or qualitatively in tissue culture and in the whole plant (Staba 1963; Krikorian and Steward 1969). In certain cases it has been observed that differentiation helps in the production of metabolites (Krikorian and Steward 1969).

The alkaloids were the earliest secondary plant products to attract attention due to their medicinal value. Glycosides, steroids, antibiotics, essential oils, and plant pigments were also isolated and studied. The plant products isolated from tissue cultures and the effect of hormones, environmental conditions and precursors on their formation will be discussed in the following pages and some of the data summarised at the end of the section (Table 2).

Alkaloids

West and Mika (1957) working with Atropa belladona cultures found that only isolated roots and root callus could synthesise atropine. Stem and leaf callus could not synthesise it unless

Table - 2

Secondary plant products isolated from plant tissue culture

No.	Secondary products isolated from tissue culture	Culture	Factors affecting the production	Reference
1	2	3	4	5
	ALKALOIDS			
1	Atropine	Excised root & root callus of A. belladena and Datura metel callus	Root or root callus is a must	West & Mika (1957); Khanna and Nag (1972).
2	Ajamalicine	Catharanthus roseus		Patterson & Carew (1969)
3	Aspidosperma	"		"
4	Iboga	"		"
5	Strychinine	"		"
6	Yohimbine	"		"
7	I, II & III (unknown)	"		"
8	Caffeine	Tea callus cultures Caffea arabica		Ogutuga & Northcote(1970); Killer et al.(1972)
9	Nicotine	N. tabacum & N.glauca or root & callus culture	IAA is needed	Furuya et al.(1967)
10	Anatabin	"		"
11	Scopolamine	D. stromonium	Tropic acid	Stohs (1969)

Table 2 contd.

1	2	3	4	5
12	Hyoscyamine	N. tabacum & D. stramonium	Tropic acid is precursor	Stohs (1969)
13	Anabasine	N. gluca		Furuya et al.(1971)
14	Harmine	Peganum harmala callus cultures		Reinhard et al. (1968a)
15	Ricinine	Excised root R. communis	Nicotinic acid & succinic acid are precursor	Hadwiger & Walter (1964)
16	Reserpine	Alstonia constricta Rauvolfia		Carew (1965); Mitra & Kaul(1964)
17	Skythanthine	Skytanthus acutus callus cultures		Luchetti (1965)
18	Indole alkaloids	Ipomea violacea, Rivea corymbosa & Argyreia nervosa suspension cultures	Mevalonic acid & L-tryptophon precursors	Dobberstein & Staba(1969)
		Rauwolfia serpentina	-NAA stimulates & 2,4D inhibits	Nikolaeva & Vollosovich(1972)
19	Alkaloids	Macleaya cordata	Highly different cells	Neumann (1968)
20	Alkaloids	N. tabacum	The synthesis is stimulated by growth inhibitors and also due to root formation	Neumann & Muller (1971)
21	Harmain Nor-Harmain	Phaseolus vulgaris	Tryptophan	Veliky (1972)

1	2	3	4	5
22	Berberine	Coplis japonica		Furuya et al.(1972); Furuya & Ishii (1972)
23	Tomatine	Tomato roots		Roddick & Butcher (1972)
24	Skimmianine	R. graveolens	Light	Steck et al.(1972)
25	Kokusaginine	"		"
26	6-OCH_3 dictamnine	"		"
27	Edulinine	"		"
28	Furoquinoline	"		Boulanger et al.(1973)
29	Solasonine	S. xanthocarpum		Hebel et al.(1968) a
30	Norsanguinarine	Papaver somniferum		Furuya et al.(1972) c
31	6-Acetonyldihydro-sanguinarine	"		"
32	Sanguinarine	"		"
33	Dihydrosanguinarine	"		"
34	Oxysanguinarine	"		"
35	Protopine	"		"
36	Cryptopine	"		"
37	Magnoflorine	"		"
38	Tropane alkaloids	Scopolia parviflora		Tabata et al.(1972)
39	Alkaloids	D. purpurea, V.minor A. belladona		Petiard et al.(1972)

Table 2 contd.

1	2	3	4	5
STEROIDS & STEROLS				
1	Phytosterols	Tobacco tissue culture *Dioscorea tokoro*	2,4D essential	Furuya *et al.*(1967)
2	Cholesterol	*Dioscorea tokoro*		Yutaka *et al.*(1970)
3	Diosgenenin	*D. deltoidea* *D. tokoro*		Kaul *et al.*(1969); Khanna & Suchendru(1973)
4	Tokorogenin	*D. tokoro*		Yutaka *et al.*(1970)
5	Yonogenin	*D. tokoro*		"
6	-Sitosterol	*Solanum xanthocaprum*		Hebel (1967); Yavagawa *et al.* (1972)
7	4,4'-Dimethyl sterol	Tobacco tissues		Benveniste *et al.*(1966a & b)
8	4-Methyl sterol	"		"
9	Canpesterol	Rice callus culture		Yavagava *et al.* (1972)
10	Stigmasterol	"		"
11	24-Methylene cholesterol	*Holarrhena antidysenterica*		Hebbel *et al.*(1971)
12	Cycloeucalenol	Tobacco		Benveniste (1968)
13	Obtusifoliol	Callus culture		"

Table 2 contd.

1	2	3	4	5
	GLYCOSIDES			
1	Scopolin	N. tabacum		Sargent and Skoog (1961)
2	Fabitrin			"
3	-Genetobioside			"
4	Unknown glycoside of scopoletin			"
5	Isosalicin	Agrostemmo githago Datura ferox		Pilgrin (1970)
6	Helicin	Agrostemina githago Datura ferox		Pilgrin (1970)
7	2-Phenethyl glucosinolate	Reseda luteola		Kirkland et al.(1971)
8	2-OH-2-Phenenthyl glucosinolate	Tropaeolum mayus		"
	VOLATILE OILS & TERPENES			
1	Geraniol	Coriandrum sativum	Illuminated cultures. in darkness it gives corresponding acetates	R Sardesai & Tipnis (1969)
2	Essential oil, rich in Ketones Methyl heptyl ketone Methyl nonyl ketone	Ruta graveolens		Reinhard et al. (1968) Cordaunt and Reinhard (1972)
3	Three new sesquiterpene lactones	Andrographis paniculata		Allison et al.(1968)
4	Cycloartenol	Tobacco tissue culture		Benveniste et al.(1966) a b

Table 2 contd.

1	2	3	4	5
5	24-Methylene cycloartenol	Tobacco tissue culture		Benveniste et al.(1966) a.&b
6	Citrostadienol	"		"
7	28-Norcitrostadienol	"		"
8	Squalene	"		Benveniste [illegible]
9	Squalene 2,3-eposcide	"		Mpang-Westrop (1967)
10	Arundoin	Rice tissue culture		Yavagava et al. [illegible] (1972)
11	Maslinic acid			Tomita and Seo (1973)
12	3-Epimaslinic acid	Isodon japonica		"
PHENOLICS				
1	Cinnamic acid	7 plant species		Netein and Jacqueline(1973)
2	p OH benzoic acid			"
3	Chloragenic acid	Potato cell suspension culture	Quinic & caffeic acid as precursor	Gamborg (1967)
4	Lignin	"		"
5	Lignin	P. nigra branch		Vanverloo (1969)
6	Lignin	Spruce bark	Coniferine precursor	Hartel et al.(1958)
7	Lignin	Scorzonera hispanica	Coniferin	Gautheret et al.(1958)
		Crataegus monogyna		
		Vitis vinifera		Hasegawa et al.(1960)
		Sequoia gigantea		
		Helianthus tuberousus		
		Pinus strobus		

Table 2 contd.

1	2	3	4	5
8	Lipids	*Kalanchoe cranata*	No sulpholipid light; glycolipid increases	Thomas & Stobart(1970)
9	Lipid constituents Triglycerides, palmitic acid & psitostenol	*Euphorbia escula*		Lee & Starall (1972)
10	Lipids - contg. sat. acids	Flax endosperm	Linoleic & linolenic acid increases under illumination	Davydova & Butenko(1968)
11	Tannin	*Juniperus communis*	Cinnamic acid precursors	Constabel (1965)
12	Pinosylvin & pinosylvin mono-methyl ether	*Pinus resinosa*	Dessiccation	Jorgensen & Balsillie (1969)
13	Catechin	Tea callus	Light synthesis of anthocyanins	& Forest (1969)
14	Leucoanthocyanins	" & *A.pseudoplatanus*		" & Goldstein *et al.*(1962)
15	Anthocyanin	~~Carrot~~ Corn endosperm	2,4D does not give anthocyanin	Straus (1960)
16	Anthocyanin	Carrot		Sugano & Hayashi(1967)
17	Anthocyanin	*Dimorphotheca auriculata*	o-Chlorophenoxy acetic acid	Ball (1970)
18	Cyanidin-glycoside	*J. artichoke*		Ibrahim *et al.*(1971)
19	Malvidin-glycoside	Carrot		"
20	Cyanidin-3,5-diglucoside	Flax		"
21	Cyanidin	*Virginia* creeper		Cronenberger *et al* (1955)

Table 2 contd.

1	2	3	4	5
22	De-oxy isoflevone (diadzin)	G. max	2,4D alone can synthesise. Citric acid promotes. Sugar concentration is critical factor.	Miller(1969, 1972)
23	Apigenin	"		Mahlbroek (1972)
24	4-CH digitolutein	Digitalis lanata		Furuya & Kojima (1971)
25	Digitolutein	"		Furuya et al.(1972b)
26	3-CH_3 quinizerin	"		"
27	Pachybasin	"		"
28	3-CH_3 alizarin	"		"
29	3-CH_3 purpurin	"		"
30	Damancanthal	Morinda citrifolia		Leistner (1973)a
31	Alizarin	"		"
32	Morindone	"		"
33	Chrysophanol	Cassia angustifolia		Friedrich & Baier(1973)
34	Physicon	"		"
35	Rhexim emodin	"		"
36	Aloe emodin	"		"
37	Rhein	"		"
38	3-Glycosides of pelargonidin, cyanidin, delphinidin	Eucalyptus citriodora		Hayata et al.(1971)

Table 2 contd.

1	2	3	4	5
MISCELLANEOUS COMPOUNDS				
1	Coumarine	Melilotus callus culture		Routein & Nichell(1956)
2	Scopoletin	N. tabaccum		Fritig et al.(1970); Reinhard et al.(1967).
3	Visnagin	Ammi visnaga		Staba et al.(1969).
4	Maackiain	Sophora angustifolia		Furuya and Ikuta (1968).
5	1-Pierocarpin	"		"
6	Pisatin	Pisum sativum		Bailey (1970)

microscopic adventitious roots had been formed. Excised roots grown in vitro had nearly the same amount of alkaloid as the root callus culture. Mitra (1972) reported 0.41 to 0.45% of atropine per 100 g. dry weight of a viable culture of excised belladona roots obtained from X-ray irradiated seeds. Chan and Staba (1965) observed that Datura stramonium callus cultures produced maximum alkaloid when grown in Murashige and Skoog's medium supplemented with coconut milk and phenylalanine. Alkaloids were present in lower concentration in root callus than in seed callus. Scopolamine was found to be present in D. stromonium suspension culture when supplied with tropic acid and N. tabacum cultures (Stohs 1969).

Nicotine, anatabine and anabasine (Furuya, Kojima and Syono 1971d) have been found to be produced by Nicotiana spp. Solt, Dawson and Christmann (1960) found that in excised root cultures of N. glauca nicotine production was proportional to root dry weight, whereas anabasine production was proportional to the product of the root dry weight and the length of the culture period. The nicotine produced by cell and callus culture was much less (0.1 ug/mg dry weight; maximum observed 7 ug/mg dry weight) than in the case of the intact plant (29 ug/mg dry weight). Krikorian (1966) has shown that callus from embryos of N. rustica contained 0.85 g of alkaloids per 100 g. dry weight. In general other species and varieties of Nicotiana tested for alkaloid gave negative or slightly positive results. Furuya, Kojima and Syono (1967) have observed that with Nicotiana callus IAA favours formation of anatabine and nicotine, but 2,4D stimulates phytosterol synthesis. Tabata, Yomamoto,

Hiraoka, Marumoto and Konoshima (1971) have reported the inhibition of alkaloid production without affecting growth of N. tabaccum callus by auxins including IAA even in the presence of Kinetin. Media containing kinetin alone increased nicotine production after a lag phase. Furuya, Kojima and Syono (1971a) have recently reported that IAA activates the biosynthesis of nicotine while 2,4D suppresses it. Anatabine and anabasine were also formed in the IAA grown callus. Neumann and Muller (1971) have reported that alkaloid formation in tobacco callus culture takes place after root formation. They also observed that growth inhibition stimulates alkaloid synthesis regardless of precursor accumulation.

Catharanthus roseus contains 66 alkaloids of which four are found to have antitumour activity (Krikorian and Steward 1969). Several workers have grown normal tissue of C. roseus (Krikorian and Steward 1969). Babcock and Carew (1962) have shown that C. roseus contains alkaloids similar in chromatographic behaviour to seed extract. Richert, Stolle, Groger and Mothes (1965) have identified vindoline and vindolinine in sten and leaf callus cultures; root callus did not contain either substance. Ajamalicine was detected by Krikorian and Steward (1965). They also observed that with different auxins, NAA and 2,4D, the alkaloids produced were different. Patterson and Carew (1969) have isolated alkaloids produced by a suspension culture of C. roseus and classified them into seven structural types.

Reserpine was isolated from the cultures of Alstonia constricta (Carew 1965) and Rauvolfia (Mitra and Kaul 1964) in small amounts. Ricinus communis excised roots produced ricinine (Hadwiger and Walter 1964). By feeding labelled nicotinic acid and succinic acid it was proved that ricinine is formed from these precursors but their concentration did not affect the amount of ricinine produced. Dobberstein and Staba (1969) have studied the influence of various chemical factors on the production of indole alkaloids by Ipomoea violacea, Rivea corymbosa and Argyreia nervosa suspension cultures. They noted that highest alkaloid production was obtained with Ipomoea cultures when grown on a medium containing alkaloid precursors like mevalonic acid and L-tryptophan. Hippeastrum vittatum (Suhadolnic 1964) callus from germinated seeds has an alkaloid different from that of the seeds. Skytanthus acutus Skythanthine hasbeen isolated from the callus culture of Skytanthus acutus (Luchetti 1965) and harmine from the stem callus of Peganum harmala (Reinhard, Corduan and Volk, 1968a). Veliky (1972) observed that cell suspension culture of Phaseolus vulgaris transforms tryptophan into harman and norharman. Neumann (1968) has shown that only highly differentiated cells formed alkaloids in Macleya cordata callus cultures. Ogutuga and Northcote (1970) detected caffeine in tea callus culture. Solasonine, a steroidal alkaloid was detected in Solanum xanthocarpum shoot callus culture (Heble, Narayanaswami and Chadha 1968). Ruta graveolens suspension culture grown in continuous light gave four alkaloids, skimmianine, kokusaginine, 6-methoxy dectamnine and edulinine (Steck, Bailey, Shyluk

and Gamborg 1971a). Kovacs, Wakkary and Goodfriend (1964) first reported the presence of a compound resembling tomatine from tomato grown gall callus. Roddick and Butcher (1972) reported the presence of tomatine in callus and excised roots of tomato. From a two year old callus tissue of Coplis japonica berberine was isolated by Furuya, Syono and Ikuta (1972a).

Glycosides

Glycosides are widely distributed in plants and some are often responsible for the taste or flavour of some foods and are also often used medicinally.

Sargent and Skoog (1961) isolated and identified scopoletin glycosides, namely scopolin, fabitrin and β-gentiobioside and an unknown glycoside which gave scopoletin, glucose and an unknown compound on acid hydrolysis. Montaldi and Skoog (1961) found that the amount of free scopoletin depends on the concentration of auxin, IAA or NAA in the medium and at a toxic level of auxin it is high. Kinetin prevented the release of free scopoletin into the medium even at a high level of auxin. Fritig, Hirth and Ourisson (1967) have shown that ^{14}C phenylalanine and tyrosine were incorporated into scopolin and scopoletin and that the synthesis of scopoletin occurred prior to the synthesis of scopolin. Pilgrim (1970) detected helicin and isosalicin in callus culture of Agrostemmu githago and Datura ferox, when fed with salicylic acid or salicylaldehyde. Kirkland, Matsuo and Underhill (1971) have reported the presence of 2-phenethyl glucosinolate from cell culture of Reseda luteola and 2-hydroxy,

2-phenethyl-glucosinolate from Tropaeolum majus cell culture. Myrosinase, a glucosinolate hydrolysing enzyme has been detected by them in seven plant tissue cultures examined. Sinapis alba callus culture contained glucosyl transferase, an enzyme associated with glucosinate biosynthesis, although no glucosinate was detected. Ibrahim, Thakur and Parmanand (1971) have shown the presence of glucosides of cyanidin and malvidin in cultures of Jerusalem artichoke and carrot respectively. They also demonstrated the presence of cyanidin-3',5'-diglucoside in flax callus culture, while the whole plant is rich in malvidin and hirsutidin.

Steroids

Benveniste, Hirth and Ourisson (1964, 1966a, 1966b) have identified campesterol, β-sitosterol, stigmasterol, 4,4'-dimethyl sterol and 4-methyl sterol in tobacco tissue culture. Benveniste (1968) also identified cycloeucalenol and obtusifoliol in tobacco callus culture and suggested that these compounds might be intermediates in sterol biosynthesis. By using labeled acetate it was shown that cycloartenol was converted to sterol possibly by more than one route (Benveniste, Hewlins and Fritig 1969). Dioscorea tokoro (Tomita, Uomori, Minato and Hitoshi 1970) and rice callus cultures (Yayagawa, Kato, Kitahara and Kato 1972) contain phytosterols, e.g. β-sitosterol, stigmasterol and campesterol. Cholesterol was present in D. tokoro (Tomita et al., 1970). As stated earlier, Furuya et al. (1967) demonstrated that 2,4D directs the metabolism of tobacco cells to produce phytosterols while IAA favours the

synthesis of alkaloids. Kaul and Staba (1968) have reported the presence of diosgenin in root suspension callus culture of D. deltoidea. Callus culture of Solanum xanthocarpum prpduces much higher quantities of diosgenin and β -sitosterol than the plant (Hebel, Naryanswami and Chadha 1968). S. laciniatum (Vagujfalvi, Maroti and Telenyi 1971) produces the same amounts as the plant. Tomita et al.(1970) have shown the presence of tokorogenin, diosgenin and yonogenin in the tissue culture of D. tokora.

Volatile oils and terpenoids

The absence of essential oils in callus cultures of Mentha sp., carrot, Pimpinella anisum, Coriandrum sativum, Anethum graveolens and Sium suave was noted (Krikorian and Steward 1969), whereas Andrographis paniculata produces three new sesquiterpene lactones (Allison, Butcher, Connolly and Overton 1968), identified as paniculide A, paniculide B and paniculide C (Butcher and Connolly 1971). However, the characteristic odors of plants are found in some cases when the callus develops minute plantlets or organised structures.

Recently Reinhard, Corduan and Volk (1968b) and Corduan and Reinhard (1972) showed the presence of essential oils in the culture of Ruta graveolens and demonstrated that light induced the callus to form essential oils corresponding to the leaf tissue, while darkness induced synthesis of essential oils similar to root tissue. Sardesai and Tipnis (1969) have shown the presence of geraniol in root callus of Coriandrum sativum, grown on White's medium with 15% CM,

4 ppm IAA and 4 ppm 2,4D. Benveniste, Hirth and Ourisson (1966b) have isolated the triterpenes, cycloartenol, 24-methyl cycloartenol, citrastadienol and 28-nor-citrastadienol from callus cultures of tobacco. Squalene-2-3-epoxide, and squalene were detected in tobacco. Squalene callus (Benveniste and Massy-Westropp 1967). Arundoin, a triterpene present in the root and leaf of the rice plant was also detected in the callus culture (Yamagawa et al. (1972).

Phenolics

The process of lignification has been shown to occur in vitro in vitro cultures of many plant cells. Wacek, Hartel and Meralla(1953) found that when coniferin and eugenol were supplied to callus cultures of carrot and spruce bark, lignin formation increased. Gautheret, Wacek and Meralla (1958) have shown that cultures, which had reduced or no power of lignification when supplied with coniferin and exposed to air, either started the lignification process (e.g. Vitis vinefera and Sequioia gigantea) or increased it (e.g. Scorzonera hispanica and Crataegus monogyna). Helianthus tuberosus shows lignification irrespective of the addition of coniferin. Maleic hydrazide, a growth inhibitor had no effect on lignin formation. Wacek et al.(1953) and Gautheret et al. (1958) have reported that propioguaicone-D-glucoside did not augment lignification. Higuchi and Barnoud (1964) have shown transformation of ferulic acid into coniferylak in in vitro grown cambial tissues of a wide variety of trees and they have also studied the lignin present in these callus cultures. Nasegawa, Higuchi and Ishikawa (1960) have isolated lignin from callus cultures of Pinus strobus grown on nutrient agar, and found that noncondensed

monomer was higher in the lignin of young tissue. The ultraviolet and infrared spectra show the characteristics of angiospermous and gymnospermous lignin. Tobacco callus did not incorporate tyrosine into the lignin (Dougall 1962). Working with a cell suspension culture of potato Gamborg (1967) has shown that quinic acid and caffeic acid can serve as direct precursors of chlorogenic acid. Callus culture of P. nigra (Vanverloo 1969) showed lignin which was like that of the young secondary xylem but having an acid resistant property like lignin from the phloem and old secondary xylem of the tree. The P. nigra callus had tracheids and more syringaldehyde/vanillin ratio when grown on IAA than on 2,4D. Schafer and Wender (1970) have studied the effect of Ca on lignification and suggested that the scopolin/scopolein ratio may be of even greater significance than either alone with regard to lignification. Koblitz (1962, 1966) reported that kinetin and giblerellin increased lignification. According to Bergmann (1964) this is due to increased number of tracheids in colonies of callus culture. Bergmann (1964) also suggested that kinetin shifts carbohydrate metabolism towards shikimic acid synthesis and accumulation of phenylpropane derivatives. Sugar level has been found to influence formation of phloem and xylem (Wetmore and Rier 1963) and also lignification (Młodzianowski 1965). In cambial callus culture of Salix cinerea kinetin IAA and NAA blocked lignification although fibrovascular bundles were formed (Saussay 1967). Wacek et al.(1953) have shown that coniferine did not form tracheids and vascular elements but increased lignification in carrot callus cultures. Thus it seems

that lignin synthesis and differentiation into lignified cells are physiologically independent.

Tannin production in callus cultures of Juniperus communis was studied by Constabel (1965, 1968). Tannin level in callus diminished with increase in dry weight or growth rate. Cinnamic acid at 10^{-5}M concentration stimulated growth and at higher level increased tannin production. Light reduced tannin content to half of that of cultures grown in darkness although nitrogen content or dry weight were not affected by it. Glucose concentration at 1-2% increased tannin content sharply, at 2-6% less rapidly, whereas at more than 6% the increase was as rapid as at 1-2%.

Many pigmented strains have been noted in tissue cultures (Gautheret 1959; Straus 1960 and Constabel 1967). Straus (1958,1960) isolated several sublines of corn endosperm which varied in their ability to synthesize anthocyanin and observed that aspartic acid enhances its synthesis. Blakely and Steward (1961, 1964) working with a highly pigmented strain of Haplopappus gracilis grown on CM, NAA and casein hydrolysate have shown that increase in auxin level inhibited anthocyanin synthesis. Blakely isolated three green strains by plating the pigmented cells on medium containing CM, NAA and casein hydrolysate. Sugano and Hayashi (1967, 1968) working with red carrot (kintoki) tissue culture found two types, one (AGI) having prominent synthetic capacity for anthocyanin and the other (AGID) which produced small amount of hydroxy cinnamic acid. IAA stimulated anthocyanin formation while 2,4D did not. Moreover, the activity of

ammonialyase for phenylalanine and tyrosine was found to be much higher in AGID than AGI. Thus it seems that the action of auxin has a direct bearing on the primary metabolic pool. Callus tissue of Dimorphotheca auriculata grown with O-chlorophenoxy acetic acid produced anthocyanins. Normal plastids were not formed when these cells were transformed to a medium containing auxin which supports the development of chloroplast in originally isolated stem tissue. Callus tissue of D. sinuata (Ball, Harborne and Arditti 1972) grown on O-chlorophenoxy acetic acid produced cyanidin-3-glucoside and delphidin-3-glucoside. Constabel, Shyluk and Gamborg (1971) have shown that H. gracilis in the presence of 2,4D accumulates anthocyanins. In the absence of auxin with kinetin or benzyl-adenine for six days the anthocyanin content of cells increased and at a higher level of NAA it decreased. Leucoanthocyanin was detected by Goldstein, Swain and Tyhio (1962) in Acer pseudoplatanus suspension culture and it was noted that a restricted amount of air reduced the synthesis. Forrest (1969) working with tea callus found that light inhibited polymerisation of leucoanthocyanin and detected catechin in the callus. Cronenberger, Vallet, Netein and Mentzer (1955) have isolated cyanidin with a trace of what was possibly delphinidin from callus culture of Virginia creeper. A colourless precursor, presumably leuccanthocyanidin (5-7-3'-4'-tetrahydroxy-flavon 3-4 diol) was also isolated.

The absence of flavanoids in the culture of mature juice vesicles from citrus fruits was earlier reported by various workers

(Krikorian and Steward 1969). Kordan and Morgenstern (1962) reported that cultured vesicle stalks from mature lemon fruits frequently released a white alcohol soluble strongly fluorescent compound. The tissue turned yellow when exposed to ammonia suggesting the presence of flavones, flavanones, chalcones and xanthones. The chromatographic patterns of young seedlings and in vitro cultures were different. Miller (1969) has isolated diadzein (deoxyisoflavone) from callus tissue of Glycine max (soyabean). In the presence of 2,4D cytokinins were not required for deoxyisoflavone synthesis. Malonic acid was more effective than citric acid in promoting synthesis of the compound. When the tissues were supplied with 0.1 M sucrose in the presence of cytokinin they formed diadzein but in the presence of 0.6 M sucrose or mannitol they formed an unidentified compound (Miller 1972). Inhibitors of protein and RNA synthesis reduced the production of diadzein but cycloheximide and puromycin promoted the synthesis of an unidentified compound. Illuminated cultures of G. max produced the flavone apigenin at the end of the growth period (Hahlbrock 1972). Berlin and Burz (1971) have isolated diadzein (I), coumesterole (II), soyagol (III) and 2',4,4'-trihydroxy chalcone (IV) from callus suspension culture of root tip of Phaseolus aureus. On prolonged culturing callus suspensions showed disaggregation and decrease in the accumulation of phenolics. β-IAA, α-NAA or kinetin promoted more of (II). In suspension culture (I) was mainly bound to a polymeric ethanolic insoluble material, although a small amount was transferred to II and CO_2.

Recently a new chalcone, echinatin, was isolated and identified by spectral analysis from the tissue culture of Glycyrrhiza echinata (Furuya, Matsumoto and Hikichi 1971b). Neitein and Jacquelin (1971) have reported the presence of simple phenolic acids, cinnamic and p-OH benzoic acid, and the absence of complex phenolic acids in tissue cultures of seven plant species studied. Jorgensen and Balsillie (1969) isolated pinosylvin and its monomethyl ether from callus culture of Pinus resinosa subjected to desiccation leading to death over a period of 3 days at 25°C. This indicates that these compounds are formed by a change in metabolism of cells dying slowly under the influence of adverse environmental conditions as postulated by Jorgensen (1961). Ulmus americana (Jorgenson and Balsillie 1969) callus culture under similar conditions formed heartwood phenols in young undifferentiated parenchymatous cells.

Furuya and Kojima (1971) first reported the presence of anthraquinones from callus cultures of Digitalis lanata. The pigments isolated were (1) digitolutein, (2) 4-hydroxy digitolutein, and later (3) 3-methyl quinizarin, (4) pachybasin, (5) 3-methyl alizarin and (6) 3-methyl purpurine were isolated and identified by Furuya, Kojima and Katsuta (1972b), the last being reported for the first time.

Miscellaneous Compounds

Visnagin, a furanochromone, has been isolated from suspension culture of Ammi visnaga (Kaul and Staba 1965, 1967). A large amount of l-maackiain and a small amount of l-pterocarpin have been isolated and identified from callus tissue of Sophora angustifolia (Furuya and Ikuta 1968). Coumarins were formed in the

cultures of Melilotus sp. (Routein and Nickell 1956). From the suspension culture of Ruta graveolens (Steck et al. (1971) when grown under continuous light a new natural product rutacultin 6,7-dimethoxy-3(1',1'-dimethyl allyl) coumarin and five coumarins, namely umbelliferone, scopoletin, psoralen, xanthotoxin, isopempinllin and rutamarin were isolated. From the root tissue culture of R. graveolens, 7,8-dimethoxy-3(1',1'-dimethyl allyl) coumarin psoralen, bergapten and xanthotoxin were identified with the help of paper and gas liquid chromatography analysis by Kuzovkina, Kuznetsova and Smirnova (1971). The phytoalexin pisatin was produced by callus culture of Pisum sativum (Bailey 1970). On prolonged sub-culture pisatin formation was reduced and it was found to be inhibitory for growth. Glycine max grown on Heller's medium with NAF (10^{-3}M) and tryptophan (1 ppm) gave fluoroacetate and fluorocitrate (Peters and Shorthouse 1972).

SECTION 3

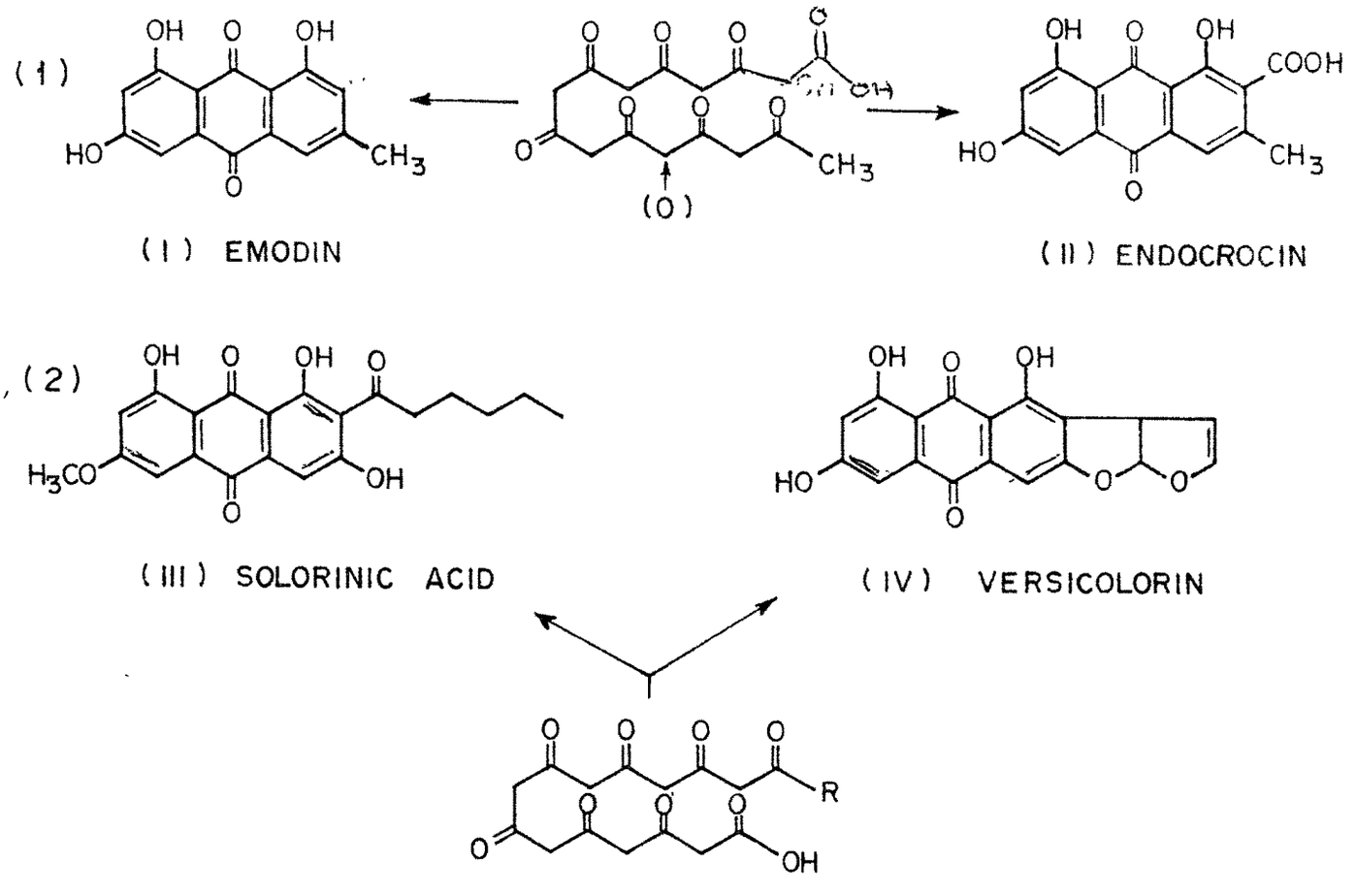
Biogenesis

Biogenesis of Anthraquinones

Most of the anthraquinones are polyhydroxy (methoxy) derivatives with little variation in skeletal structure. Early analysis of the natural anthraquinones by Birch and Donovan(1953,1955) suggested that there are at least two biosynthetic routes for the formation of anthraquinones, (a) compounds like emodin (I) had structures in accord with the acetate hypothesis, (b) while a group of compounds related to alizarin (IX) seemed to be formed in some other way, i.e. by shikimate and mevalonate route as it was later proved by Zenk and Listner (1967a, 1968a and 1968b) and Burnett and Thomson (1967 and 1968c).

Labelling experiments with ^{14}C acetate by Birch, Ryan and Smith (1958) and by Gatenbeck (1958 and 1960) established the acetate derivation of heminthosporin, emodin, islandicin and cynodontin. Additional confirmation was obtained by using ^{14}C, ^{18}O acetate as precursor. Gatenbeck (1960), Bentley and Keil (1961), Bu'lock and Smalley (1961) and Bu'lock, Smalley and Smith (1962) showed that aromatic polyketides were actually built up from a starter unit, usually acetate, and a chain of malonate units (formed by carboxylation of acetyl COA). This was confirmed in the case of islandicin (Gatenbeck 1962) and the bianthraquinone rugulosin (Shibata and Ikekawa 1962, 1963). These results were obtained using mols and since all fungal anthraquinones are structurally consistent with the acetate-mevalonate route this conclusion appears reasonable.

FIG. 1

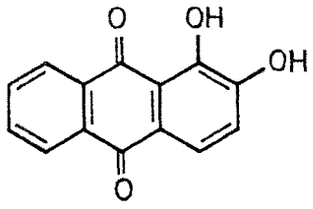


BIOGENESIS OF ANTHRAQUINONES FROM ACETATE

As shown in Fig. 1 the variations in carbon skeleton can be attributed to folding of the polyketide chain in different ways prior to cyclisation, and also resulting from O-methylation, side-chain oxidation dimerisation and the introduction or omission of nuclear hydroxyl groups. The presence of two β-methyl groups in the pigment of Curvularia spp. is exceptional, the origin of the second being a matter for conjecture. The products of animal metabolism have α-side chain in their anthraquinone molecule (an exception being Eriococcus confusus which contains emodin and 7-hydroxy emodin). There are indications that phenolic compounds found in marine invertebrates arise by the acetate malonate pathway, although biosynthesis by microbial flora cannot be excluded (Salaque, Barbier and Lederer 1967).

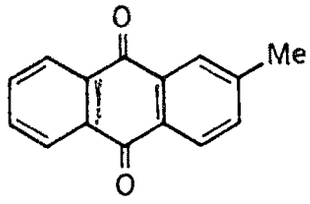
Half of the anthraquinones found in higher plants unlike emodin are substituted in only one oft the benzenoid ring and may be totally devoid of a carbon side chain, i.e. alizarine (IX) or hydroxyl groups, i.e. tectoquinone (X). The majority of these occur in the Rubiaceae (subfamily-Rubioideae) and, to a lesser extent, in the Bignoniaceae and Verbenaceae (teaks) heartwood, tectoquinone (X) being present in all three. The anthraquinones present in Bignoniaceae and Verbenaceae (Burnett and Thomson 1967, 1968)/(Sandermann and Simatupang 1966) heartwood are accompanied by C_{15} naphthaquinones, ex.deaxylapachol (XI), while the Rubiaceous plants contain a number of C_{15} naphthalenic compounds)XII), (XIII) and (XIV) and also 4-methoxy-1-naphthol (Burnett and Thomson 1968b). These finds suggested that (XI) is synthesised in vivo by prenylation of a naphthol precursor followed

FIG. – 2.



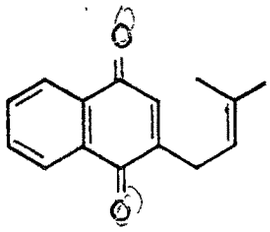
(IX)

ALIZARIN



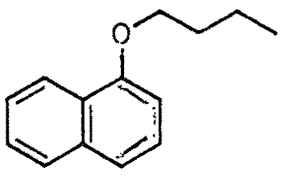
(X)

TECTOQUINONE

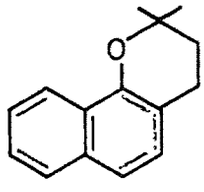


(XI)

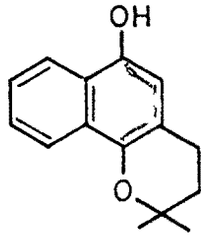
DEOXY LAPACHOL



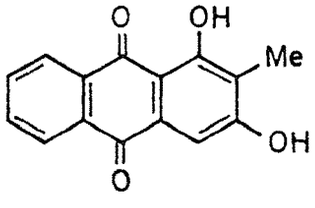
(XII)



(XIII)

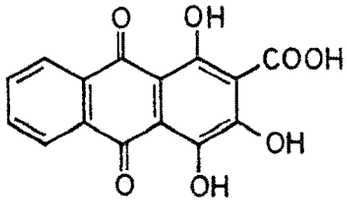


(XIV)



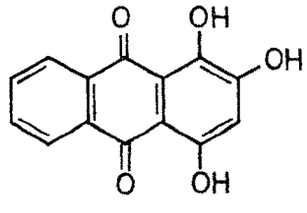
(XV)

RUBIADIN



(XVI)

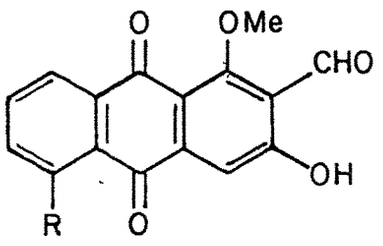
PSEUDOPURPURIN



(XVII)

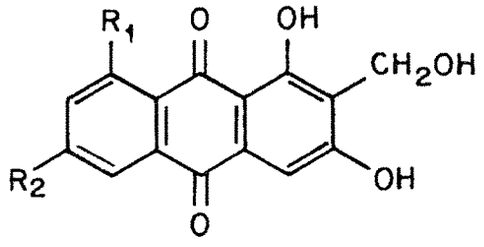
PURPURIN

FIGURE-3



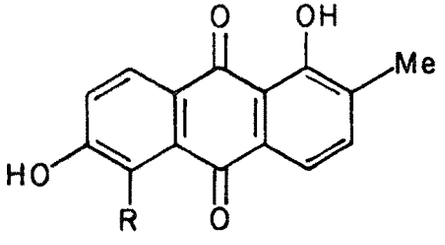
(XIX)

R = OH JUZUNAL } Damnacanthus majar
R = H DAMNACANTHAL }



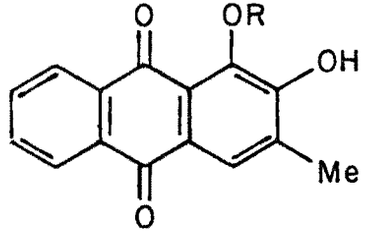
(XX)

R_1 = H, R_2 = OH - COLUCIDIN Copsosma lucida
R_1 = R_2 = H - LUCIDIN
R_1 = OH, R_2 = H - COELULATIN } Coelospermum



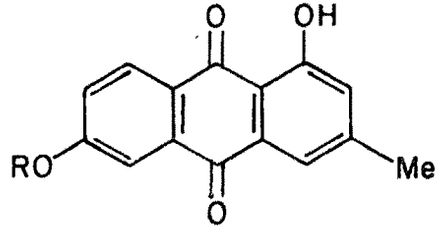
(XXI)

R = OH - MORINDONE
R = H - SORANJIDIOL } Marinda spp.



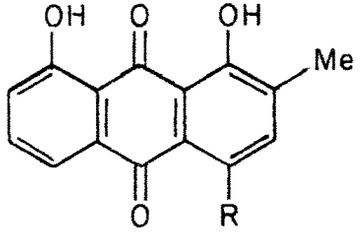
(XXII)

R = H — 3 — METHYL ALIZARIN
R = CH_3 - DIGITOLUTEIN } Digitalis spp



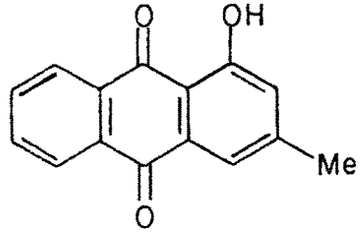
(XXIII)

R = H, OH
From Digitals purpurea



(XXIV)

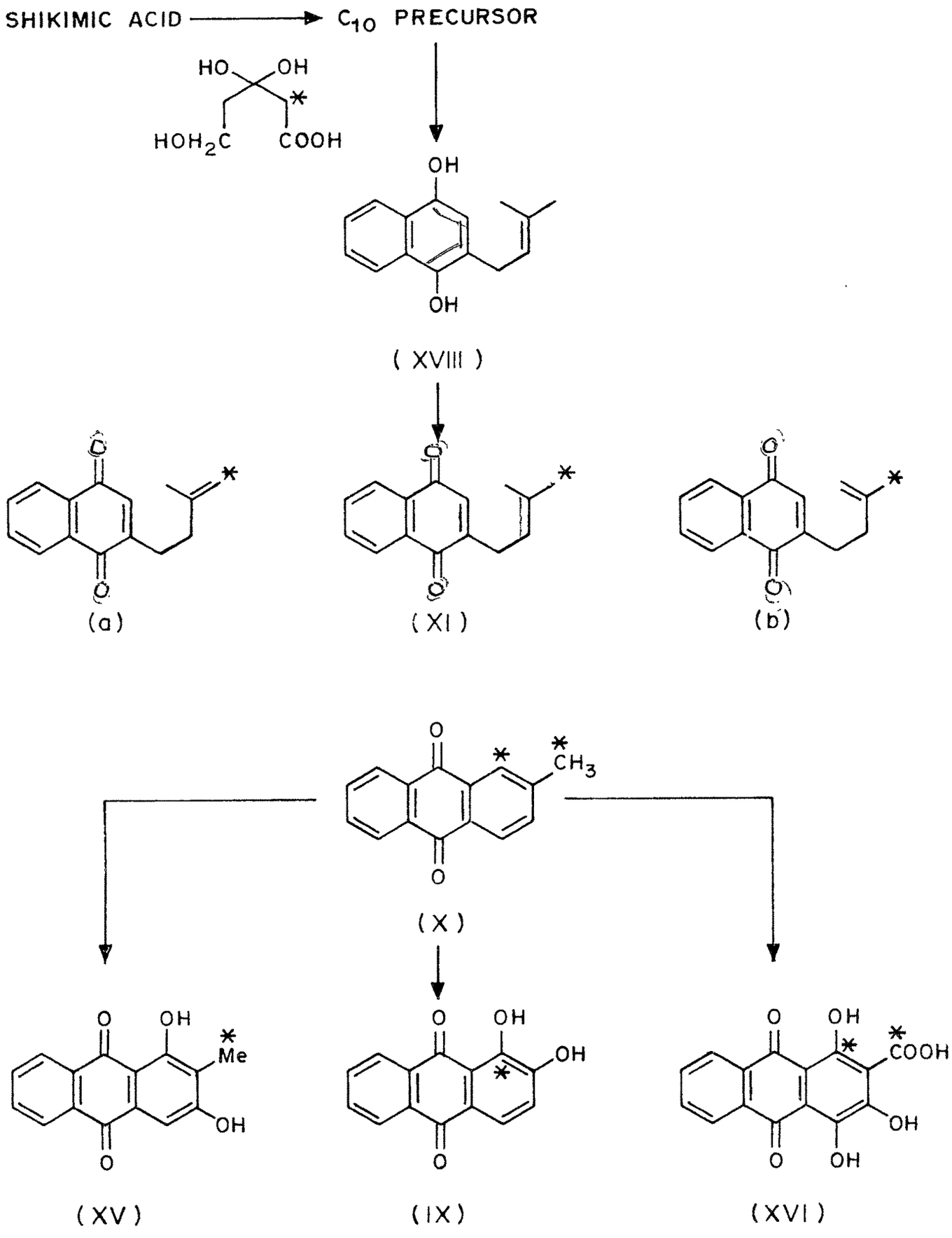
R = OH or H From D. Purpurea



(XXV) PACHYBASIN

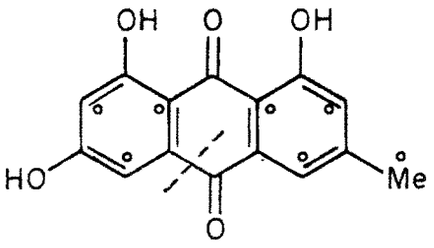
by oxidation, and since (XI) can be converted to (X) *in vitro*, either by borontrifluoride catalysis or by irradiation, it seems likely that the substituted (C) ring in this group of anthraquinones is derived from mevalonate. Burnett and Thomson (1968c) and Listner and Zenk(1968a) have established the above hypothesis by feeding experiments in *Rubia tinctorum* (madder) plants with 2-^{14}C mevalonate. Appropriate degradations of pseudopurpurin (XVI), which had the same specific activity as rubiadin (XV) and double that of alizarin (IX) and purpurin (XVII) established that carbon-14 was distributed between the side-chain and C_1 in ring (C). This indicates that the origin of ring C in Rubiaceous anthraquinones is as shown in scheme 1 and presumably the same pathway is followed in Bignoniaceae and Verbenaceae. The C_{15} intermediates (XI and XVIII) have not been detected in *R. tinctorum* but a pyran (XIV) has been isolated which is equivalent to (XI) and (XVIII), and a low incorporation of deoxylapachol (XI) into alizarin has been effected. The C_{10} precursor has not been isolated and identified. Labelled 1,4-naphthaquinone was readily incorporated into alizarin (Leistner and Zenk 1968c), most probably by reduction and prenylation of the quinol. U-^{14}C shikimic acid has been shown to be totally incorporated into alizarin and purpurin (Leistner and Zenk 1967b) in *R. tinctorum* and thereby provides the whole of ring A and one of the quinone carbonyl groups. Hence the C_{10} precursor must have originated from shikimic acid.

All the anthraquinones in *Rubiaceae* are not substituted in one ring only. As shown in Fig. 3, some of the species (Coprosma, Morinda, Digitalis,etc.) contain both one ring substituted and both the ring substituted anthraquinones. These can be derived from the shikimate-mevalonate pathway, but there is no experimental evidence.



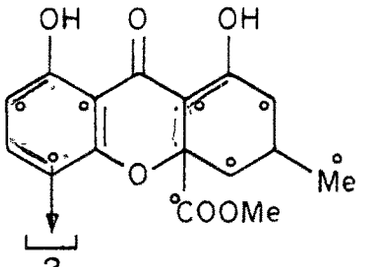
SCHEME—1

BIOGONESIS OF RUBIACEAE ANTHRAQUINONES



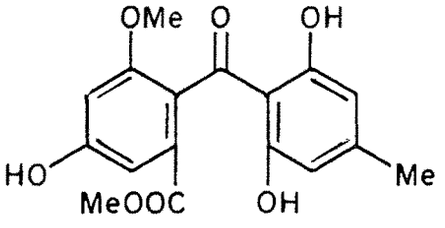
(I)

EMODIN Claviceps purpurea



(XXVI)

ERGOCHROME



(XXVII)

SULOCHRIN

in Penicillium frequentans

Digitalis was known to have only one ring substituted anthraquinone, leading to the conclusion that they are derived by shikimate-mevalonate pathway. However, in a recent investigation of D. purpurea, digitolutein and 3-methyl alizarin were absent or barely detectable among a large number of trace components of which (XXIII) and (XXIV) could be identified. Pachybasin (Curtis, Hassal and Parry 1971) an anthraquinone substituted in one ring only, which occurs in teak wood (Verbenaceae) and as a metabolic product of the fungus Phoma foveata was found to be synthesised by acetate-mevalonate pathway in the fungus. Chrysophenol (Leistner 1971) in Rumex alpinus and Rhamnus frangula was found to incorporate labelled acetate. Hence the possibility that plants may utilize more than one metabolic pathway for the formation of anthraquinone cannot be ruled out.

Until recently anthraquinones were regarded as metabolic end products. However, in ergot (Claviceps purpurea) anthraquinones and ergochomes (XXVI) occur together (Franck 1969) and it has been established that the latter are derived from the former by feeding experiments with labelled emodin. Similarly sulochrin (XXVII) was shown to be derived from questin. (1,6-Dihydroxy-3-methyl,8-methoxy-anthraquinone)in cultures of Penicillium frequentans (Thomson 1971).

Study of biogenesis in tissue cultures

In his review Staba (1963) has discussed the biosynthetic potentialities of various organ and tissue cultures and has stated that tissue cultures are cytologically, physiologically and biochemically distinct from the parent plants. However, a few organ and tissue cultures have been reported to synthesise compounds present in parent plants.

The biosynthesis of secondary metabolites was studied in plant tissue cultures by feeding various precursors. Thus by feeding L-ornithine-HCl, L-phenylalanine and both together, and from the percentage of alkaloid isolated from callus cultures of Datura stramonium, the biosynthetic pathway for tropane alkaloid was postulated (Tomita 1971). One of the earlier reports on biosynthesis of secondary metabolites by using the tracer technique was by Hasegawa, Higuchi and Ishikawa (1960) on biosynthesis of lignin. Tissue culture of white pine readily incorporated glucose-1-^{14}C and shikimate-R-^{14}C into lignin (Higuchi, Ishikawa and Hasegawa 1960). Further work by Higuchi (1962) has shown that phenyl alanine, p-coumaric acid and ferulic acid were quite good precursors of conifer lignin and that the lignification route via phenylpropanoic acid is operative in these tissues. Bardinskaya (1960) has reported the use of tissue culture in the study of biosynthesis of lignin, and Gamborg (1967) using potato cell cultures has shown the direct incorporation of ^{14}C quinic acid and ^{14}C caffeic acid into chlorogenic acid and also has shown that a large portion of labelled carbon from aromatic compounds (cinnamic acid, p-coumaric acid, caffeic acid, shikimic acid and quinic acid) was incorporated into the alcohol insoluble fraction and was associated with Klason lignin. Solt et al.(1960) using root cultures of N-glauca have shown that labelled ornithine was incorporated into nicotine and labelled lysine into anabasine. By using labelled acetate as precursor labelled squalene 2,3-epoxide was isolated from tobacco tissue culture and was shown to be an

intermediate in the biosynthesis of phytosterols (Benveniste and Mussy-Westropp 1967). ^{14}C-squalene 2,3-epoxide was converted to cycloartenol and 24-CH_3 cycloartenol by tobacco tissue culture under anaerobic conditions (Eppenberger 1969). Fritig, Hirth and Ourisson (1970) have shown the incorporation of labelled L-phenylalanine into scopoletin, and scopolin, cinnamic acid and hydroxy cinnamic acids (p-coumaric acid, caffeic acid and ferulic acid) and also indicated that scopoletin is synthesised prior to scopolin. The biosynthesis of scopoletin was studied by feeding tobacco callus cultures with U-^{14}C phenylalanine, 2-^{14}C-cinnamic acid, 2-^{14}C glucoside ferulic acid and methyl-^{14}C-methionine (Tomita 1971). Ricinine biosynthesis in excised roots of Ricinum communis was studied by Hadwiger and Waller (1964) and was found to incorporate nicotinic acid-7^{14}C and succinic acid-2,3-^{14}C. Stohs (1969) has shown the incorporation of radioactive tropine and tropic acid into scopolamine and hyoscyamine by suspension cultures of Datura stramonium and N. tabacum. It has been shown that the amount of scopolamine produced exceeded the formation of hyoscyamine and cultures of D. stramonium more than three months old lost much of their ability to synthesise these two alkaloids. Benveniste et al. (1969) has shown the non-specific conversion of ^{14}C-cycloartonol (which was obtained with other labelled sterols after feeding the callus with labelled acetic acid) into sterols and stated that the scheme, cycloartenol → methyl-24-cycloartenol → cycloeucalenol → obtusifoliol → "lophenols" etc. might be only one of the pathways followed. By adding 100 mg/l. tryptophan to the standard medium of Phaseolus vulgaris the concentration of the alkaloids norharman

and harman were increased and also no alkaloid was produced with inorganic nitrogen indicating a direct role of tryptophan in the biosynthesis of carboline alkaloids (Veliky 1972).

The conversion of labelled mevalonic acid into sapogenins, diosgenin, yonogenin and lokosogenin by the following route, in callus cultures of Datura tokoro have been reported: MVA → squalene 2,3 epoxide → cycloartenol → cholesterol → 26 hydroxy cholesterol → 16,26 dihydroxycholesterol → 16,22,26 trihydroxycholesterol → diosgenin → yonogenin → tokonogenin (Tomita 1971).

PRESENT INVESTIGATION

The object of this work is to establish tissue culture of forest trees and to study their nutrition and the formation and biosynthesis of phenolics by a tissue culture.

Viable callus cultures of Tectona grandis (Teak), Artocarpus heterophyllus (Jack), Morus alba (Mulberry) and Populus nigra (Poplar) have been established in vitro. The nutritional requirements of teak callus were studied in detail and one unknown and two known phenolic compounds were isolated and characterised. The structure of the unknown compound was established by synthesis and its biosynthesis was studied by the use of isotopically labelled precursors.

Tissue cultures of some species of poplar have been established in vitro by several workers (Gautheret 1959; Walter 1964; Mathes 1964a). The cultures of other trees, i.e. jack, mulberry and teak have been initiated and established as viable cultures for the first time in the present work.

Chapter 1 deals with Materials and Methods.

Chapter 2 deals with Initiation and establishment of continuous cultures of Teak, Mulberry, Jack and Poplar.

Chapter 3 deals with Nutritional requirements of teak tissue culture.

Chapter 4 deals with Isolation, identification and synthesis of teak quinone-A.

Chapter 5 deals with (a) Effect of some nutritional factors on teak quinone-A. (b) Biosynthesis of teak quinone-A.

CHAPTER - 1

MATERIALS AND METHODS

***

SECTION - 1

Materials

The inorganic salts used for the preparation of media and the carbohydrates were of analytical grade (British Drug House or E. Merck). The following were from the sources indicated in parenthesis. Yeast and malt extracts, bacto casamino acids, casein hydrolysate and bacto agar (Difco), auxins, vitamins, nucleotides, kinins, aminoacids (Sigma Chemical Company or BDH), AMO 1618 (California Biochemicals).

I wish to acknowledge generous gifts of the following chemicals: Zeatin from Dr. G. Shaw, U.K., and Dr. D.S. Letham, New Zealand, 2-BTOA from Dr. H.Y. Mohan Ram, New Delhi, C.C.C. from American Cyanamid Company, New Jersey, abscisic acid (II) from Hoffmann La Roche, Basle, and gibberellins (GA_1, GA_3, GA_4, GA_5, GA_4/GA_7, GA_9 and GA_{13}) from Imperial Chemical Industries, U.K.

The plant materials used are from the following sources: Tectona grandis (Teak) and Populus nigra (Poplar) from the Poona University garden. Morus alba (Mulberry) from the garden of this Laboratory. Artocarpus heterophyllus (Jack fruit). Seeds collected from freshly cut fruit from the local market and germinated as described in Methods.

Ferric ethylene diamine tetraacetic acid was prepared according to Murashige and Skoog (1962). Coconut milk was obtained by pooling together the filtered water from a number of tender

green coconuts, autoclaving at 15 lbs per sq.inch for 20 min. and storing at -20°C. Before use it was thawed and filtered.

The silica gel used for chromatography was 200 mesh for thin layer chromatography and 40-60 mesh for column chromatography. All the solvents used were distilled and purified. The spectrum pure alcohol was obtained by twice distilling alcohol, and stored in a brown bottle. Before use its purity was checked by taking its UV spectrum.

Glassware

All glassware used was Corning brand. It was cleaned by boiling in a solution of sodium carbonate, rinsing with tap water, immersing in 30% nitric acid and then washing successively with tap water, distilled water and glass distilled water. It was dried at room temperature on a draining rack. Test tubes and flasks used for cultures were plugged with absorbent cotton wool, autoclaved at 20 lbs. per sq. inch for one hour and dried at 100°C for 2 hours.

SECTION - 2

Composition of Media

The composition of the different basal media used in the course of this work is shown in Tables 3A, 3B and 3C. The supplements added to the basal media and their concentrations are described in the text. Modified Reinert and White's (1966), Murashige and Skoog (1962) and Blaydes (1966), macro and micro salts (Tables 3A and 3B) were prepared according to their original publications. All the other media were modified as described below and therefore refer not to the media described in the original publications, but to modification as shown in the respective tables and marked with an asterisk. The macro salts (Table 3A) in the media were exactly as described in the original publication. Among the micro elements (Table 3B) iron was added as ferric EDTA in White's and Smith's at the concentrations shown in the Table instead of ferric sulphate (Whites 1954) and ferric tartrate (Smith 1967). The trace elements added to Knop's were according to Smith (1967) and aluminium and nickel chlorides omitted from the micro salt solution originally used by Heller (Gautheret 1959). Copper and molybdenum were added to Whites media at the concentrations used by Smith and Smith's medium was supplemented with potassium iodide at the same level as in White's medium. Thiamine, pyridoxine, nicotinic acid and glycine (Table 3C) at a ten times higher concentration than those used by White (1954) were added in Whites, Knops, Hellers and Smiths media (Table 3C). In Murashige and Skoog's medium glycine was added at 1 mg instead of 2 mg/l and in Reinert and White's at

Table 3A

Inorganic salts in different media: Macro elements
(Concentration expressed as mg/l of media)

Chemical	White	Reinert & White *	Murashige & Skoog	Knop	Heller	Smith	Blaydes
$Ca(NO_3)_2 \cdot 4H_2O$	288	425	-	144	-	710	347
NH_4NO_3	-	50	1650	-	-	-	1000
KNO_3	80	170	1900	25	-	-	1000
$CaCl_2\ 2H_2O$	-	-	440	-	75	405	-
Na_2SO_4	200	425	-	-	-	-	-
KCl	65	140	-	-	750	-	65
$NaH_2PO_4\ 2H_2O$	22	35	-	-	125	-	-
KH_2PO_4	-	-	170	25	-	70	300
$MgSO_4\ 7H_2O$	360	764	370	51	250	245	35
NaCl	-	-	-	-	-	60	-
$NaNO_3$	-	-	-	-	600	-	-

* Water of crystallisation is not included

Table 3B

Inorganic salts in different media. Micro elements
(Concentration expressed as mg/l of medium)

Chemical	White	Reinert & White*	Murashige & Skoog	Knop	Heller	Smith	Blaydes*
$FeCl_3\ 6H_2O$	-	-	-	-	1.0	-	-
Na_2 EDTA	37.30**	37.30	37.30	37.30**	-	37.30**	223.96
$FeSO_4\ 7H_2O$	27.80**	27.80	27.80	27.80**		27.80**	116.8
$ZnSO_4\ 7H_2O$	2.60	3.2*	8.6*	0.62**	1.0	0.62	1.5
$MnSO_4\ H_2O$	5.0	9*	22.3*	-	0.1	-	4.4
$MnCl_2$	-	-	-	0.40**	-	0.40	-
H_3BO_3	1.50	3.2	6.2	0.57**	1.0	0.57	1.6
KI	0.75	1.6	0.83	0.75**	0.01	0.75**	0.8
$CuCl_2$	0.27**	-	-	0.27**	-	0.27	-
$Na_2\ MoO_4\ 2H_2O$	0.25**	-	0.25	0.25**	-	0.25	-
$CuSO_4$ - $5H_2O$	-	-	0.025	-	0.03	-	-
$CoCl_2$ - $6H_2O$	-	-	0.025	-	-	-	-

* Water of crystallisation is not included

** Changed from original medium

TABLE 3C

Organic constituents in different media

(Concentration expressed as mg/l)

Chemical	White*	Reinert & White	Murashige & Skoog	Blaydes'
Thiamine HCl	1	0.1	0.1	0.1
Pyridoxine HCl	1	0.1	0.5	0.1
Nicotinic acid	5	0.5	0.5	0.5
Glycine	30	3.0*	1.0*	2.0
Inositol	-	10.0	100	-

* Changed from original medium.

Table - 3D

Composition of basal media used for the initiation and maintenance of teak, mulberry, jack and poplar callus.

Media	Macro elements	Micro elements	Vitamin & glycine
WBM	White's (Table 3-A)	White's (Table 3B)	White's (Table 3C)
RWBM	Reinert & White's (Table 3A)	Reinert & White's (Table 3B)	Reinert & White's (Table 3C)
KBM	½ strength Knop's solution (Table 3A)	Smith's (Table 3B)	White's (Table 3C)
SBM	Smith's (Table 3A)	Smith's (Table 3B)	White's (Table 3C)
$MSBM_1$	Murashige & Skoog's (Table 3A)	Murashige & Skoog's (Table 3B)	Murashige & Skoog's (Table 3C)
MSB	"	"	" + glycine 1 ppm
BBM	Blaydes' (Table 3A)	Blaydes' (Table 3B)	Blaydes' (Table 3C)

Table 4

Maintenance media for callus cultures of teak, jack, mulberry and poplar

Medium	Tissue	Salt solution	Vitamin & glycine	Auxin, CM or kinetin	Other growth factors	Sucrose
1	Teak (branch)	Murashige & Skoog's (Tables 3A & 3B)	Murashige & Skoog's (Table 3C)	IAA 5 ppm Kinetin 1 ppm	glycine 1 ppm	3%
2	Teak(branch)	Murashige & Skoog's (Tables 3A & 3B)	Murashige & Skoog's (Table 3C)	IAA 5 ppm CM 15%	glycine 1 ppm	3%
3	Jack(seedlings)	Blaydes' (Tables 3A & 3B)	Blaydes' (Table 3C)	2,4D 1 ppm kinetin 0.5 ppm	10 ppm tyrosine 10 ppm phenyl alanine	2%
4	Mulberry(branch)	Murashige & Skoog's (Tables 3A & 3B)	Murashige & Skoog (Table 3C)	IAA 5 ppm kinetin 1 ppm	pantothenate 1 ppm biotin 0.1 ppm	3%
5	Poplar(branch)	"	"	NAA 1 ppm kinetin 1 ppm	pantothenate 0.1 ppm biotin 0.01 ppm	3%

3 mg instead of mg/l. No changes were made in the organic constituent composition in Blayde's medium.

The sucrose concentration was 20-30g/litre and that of agar 7 g/litre. The sucrose concentration used is mentioned in the composition of each medium in Chapter II for the initiation of callus, but in the nutritional studies on teak callus (Chapter III) it was kept at 30 g/litre according to Murashige and Skoog (1962).

Abbreviations have been given in Table 3D to describe the composition of the more frequently used media. Any changes in these basal media are shown in the corresponding tables.

Preparation of media

After the addition of the supplements indicated in the respective tables the basal medium was adjusted to pH 5.8-6.0 and then made to volume. For semisolid medium agar was added at 0.7%. The medium was steamed for 30 min. to melt the agar and transferred in 20 ml lots to test tubes (25 x 150 mm). Sterilization was carried out by autoclaving at 15 lbs per sq.inch for 20 min., followed by steaming for 30 min. on the subsequent day. Heat labile compounds were sterilized either by passing through a seitz bacterial filter or dissolving the solid compound in 70% alcohol and storing in a sterile flask. The heat labile compounds were added aseptically to the medium at the required levels after the autoclaved medium had been steamed on the second day and had come down to a temperature of 40°C. The contents of the tubes or flasks were then mixed thoroughly.

SECTION 3

Methods of Subculture and Growth Measurement

(a) Inoculations

All inoculations were carried out in a sterile room under an inoculation hood having an ultraviolet lamp which was switched off during inoculations. A continuous stream of air, which was sterilised by passing through aluminium strips coated with glycerine followed by irradiation with U.V. light was passed through the room. Sterile forceps, scalpels and Petri dishes were used for transferring the plant tissues during the inoculation and subculture. Callus tissue pieces of teak, mulberry, jack fruit and poplar having approximately uniform initial weight, as judged visually, were removed from four to five weeks old agar cultures and transferred to test media. Teak callus was generally grown on Medium No.1 (Table 4) for maintenance and for use as inocula for growth experiments. The wet weight of teak callus inoculum was between 80 - 120 mg. per tube. Similarly jack, mulberry and poplar callus were grown on Media 3, 4 and 5 (Table 4) respectively. The inocula sizes for these cultures were between 60-80 mg. per tube.

Measurement of growth

Wet and dry weights were taken as a measure of the growth of the callus. Dry weights were obtained by drying the callus at 100°C ± 5. In comparing growths both dry and wet weights were taken into account. Changes in dry and fresh weight were generally similar but in some cases the water content varied considerably.

Standard error

To determine the difference in weight between replicate cultures an experiment was set up in which teak callus of uniform size was transferred into 20 tubes of standard medium. After 35 days the weight of the tissue in each of the tubes was taken and the standard error was obtained by the formula $\pm\sqrt{\frac{\sum\Delta^2}{n(n-1)}}$ where Δ is the difference between the individual value and the average and n the total number of tubes (Murashige and Skoog 1962).

The difference in the weights of the inoculum was also determined in a similar manner using 20 different pieces of fairly uniform inocula. The standard error of inoculum was below 7% and for the final weight between 6 and 10 per cent. The results of experiments in which there was visibly a markedly (higher or lower) growth in one or more tubes were not taken into account for determining standard error.

The growth measurements of all the cultures were generally made at the end of 30 to 35 days after which time the wet and dry weights were determined. The average growth of five replicate cultures was measured by Steward (1963) in his growth studies and a similar number of cultures was kept in the present investigation.

Incubation of cultures

After inoculation the cultures were incubated in a dark room at 27°C ± 1°C where the relative humidity was maintained at 60 to 70 per cent.

SECTION 4

(a) Estimation of teak-quinone A

A known quantity of teak quinone A (crystallised from benzene and isolated as described in Chapter IV) was dissolved in spectrum pure alcohol and the absorption spectrum of this standard solution was recorded on a Beckman ratio recording spectrophotometer model DK-2. The pigment showed maximum absorbance at 410 mμ and 276 mμ.

Teak quinone A was isolated from teak callus grown on different media and its absorbance at 410 mμ or at 276 mμ was measured either on a Beckman spectrophotometer model DK-2 or DU. From the optical density of a known amount of sample of pure Teak-quinone A, the amount of Teak quinone-A/g tissue for the unknown samples was calculated.

(b) Counting of ^{14}C labelled teak-quinone A

Teak callus culture grown on Murashige and Skoog's medium having GA_3 at 10 ppm was fed with labelled acetate (2-^{14}C) and 2-^{14}C mevalonate. The detailed experimental procedures of feeding and isolation of pigment are described in Chapter V, Section 2.

After purifying the Teak quinone A, its concentration was estimated as described in Section 4(a). A known amount of labelled teak quinone A was dissolved in a known quantity (0.1 to 0.2 ml) of pure methanol and this was added to 5 ml of Bray's scintillation fluid of the following composition: Naphthalene 120 g%; PPO 8 g%; POPOP 0.4 g%; Methanol 200 ml; Ethylene glycol 40 ml; Dioxane 1700 ml.

The radioactive counts were taken on a Packard liquid scintillation Spectrometer, model 3002, having 90% efficiency. From the counts per mg of pigment taken, the counts per total amount of pigment was calculated. Percentage incorporation and specific activity were calculated by the following formulae:

$$(1)\quad \%\ \text{incorporation} = \frac{\text{dpm of isolated compound}}{\text{dpm of precursor fed}} \times 100$$

$$(2)\quad \text{Specific activity} = \frac{\text{dpm of compound taken for counts}}{\text{weight of the compound taken for counts}} \times \text{Molecular weight of the compound.}$$

C H A P T E R - II

INITIATION AND MAINTENANCE OF CALLUS FROM EXPLANTS OF TEAK, JACK, MULBERRY AND POPLAR

***

When this work was initiated there were very few reports regarding the establishment of viable callus cultures from forest trees. According to Gautheret (1959) only 4 out of 25 species tested formed viable callus, while Jacquiot (1959) was successful in cultivating seven out of twenty five trees tested. In most instances suboptimal nutritional conditions might have been responsible for failure to establish successful cultures from this group of trees. In order to initiate and establish viable callus cultures from teak (Tectona grandis), jack (Artocarpus heterophyllus), mulberry (Morus alba) and poplar (Populus nigra) several media were tested. The possibility that media necessary for callus initiation and for maintenance on subculture may be different was kept in view when this work was begun.

This chapter deals with the standardization of conditions for callus initiation from branch segments or seedlings of teak, jack, mulberry and poplar and studies on their viability on repeated subculture. Greater emphasis has been given to teak because of its economic importance, its ready callus formation and the visible pigmentation of the callus. Based on the results of these studies, callus cultures of the other three forest trees were obtained and the effect of different nutrient supplements tested.

SECTION 1

(a) Initiation of callus from teak

A branch 2.5 - 4 cm in diameter from a teak tree, approximately 8 years old, was cut into 5-8 cm pieces. For sterilization two methods were followed. (All operations were carried out under aseptic conditions with sterile solutions). In the first, segments of the branch were washed with distilled water after scraping off the outer bark and then rinsed for 10 min with distilled water containing a detergent (DET). The detergent solution was poured off and the pieces were washed thoroughly with distilled water, and then put into 70% alcohol for 10 min., washed with distilled water and finally transferred to a flask containing a 10% sodium hypochlorite solution. This treatment was given for 20 min. under vacuum with occasional shaking. The hypochlorite solution was then poured off and the pieces were rinsed 5-8 times with distilled water. In an alternative method of sterilization after washing with detergent, the pieces were flamed 3 times after dipping in 70% alcohol.

The sterilized stem pieces were placed separately on a sterile wooden block and 2-4 mm. square portions containing the cambium and phloem were carefully dissected out with a scalpel and then transferred to test tubes containing different media. Younger twigs (1-2 cm diameter, 2-3 cm length) after sterilization were divided longitudinally into 4 wedge shaped pieces and transferred to different media. Initiation of callus was observed after 10-15 days. In addition to branches and twigs, petioles were also used. The petioles were cut into 0.5-1 cm sections and transferred to tubes containing different media.

Table - 5

Effect of different media on callus formation from teak explants

Inoculum used : Branch segments 1" to 1½" thick and petiole ½" thick.

Basal medium : WBM & RWBM

Sucrose : 2%

Period of incubation : 25 days

Results : + 60 to 80 mg, ++ 80 to 150 mg., +++ 150 to 350 mg., ++++ more than 400 mg.

NT : Not tested

Concentration of other supplements (ppm) : NAA - 1; 2,4D - 0.6; kinetin - 1; YE - 100; ME - 100; inositol - 10; pantothenate 0.1; biotin - 0.1; casein hydrolysate(CH) - 200; Vit. C - 0.1; GA_3 - 10; CM - 15%.

No.	Basal medium	Auxin	Cytokinin	Other supplements	Results	
					Branch	Petiole
1	WBM	NAA	CM		+	NT
2	"	"	"	YE	+++	"
3	"	"	"	YE, ME	-	"
4	"	"		YE, ME, Inositol	-	"
5	"	"	"	YE, ME, Inositol, pantothenate	++	"
6	"	"	"	Pantothenate	+	"
7	"	"	"	Pantothenate, Biotin	+++	"
8	WBM	"	"	CH, vit. C, GA_3	+	"
9	"	2,4D	"	CH	+	"

Table 5 contd.

No.	Basal medium	Auxin	Cytokinin	Other supplements	Results: Branch	Results: Petiole
10	RWBM	2,4D	6M Kin	-	-	-
11	RWBM-Pyridoxine	"	"	-	+	-
12	RWBM	"	"	YE, ME, CM	+	+
13	RWBM	"	"	Pantothenate, biotin	+	+
14	"	"	"	CH	+++	++
15	"	"	"	GA_3	++	-
16	RWBM-Pyridoxine	"	"	"	++	+
17	RWBM	"	"	GA_3, vit.C	++	++
18	"	"	"	CH, GA_3, vit. C	++	+
19	RWBM-Pyridoxine	"	"	CH, Vit.C	++	NT
20	"	"	CM	CH, Vit.C	++++	"
21	RWBM-Pyridoxine	"	-		-	-

Twenty one different media combinations were tried for the initiation of callus (Table 5) White's basal medium (WBM) supplemented with yeast extract (Expt. 2) or pantothenate and biotin with NAA and coconut milk (Expt. 7), and Reinert and White's modified basal medium (RWBM) having casein hydrolysate, 2,4D and kinetin gave good callus formation. RWBM without pyridoxine and with 2,4D, CM, CH and vitamin C gave best callus formation (Expt. 20). The minimum requirement for callus formation was a basal medium containing NAA and CM or RWBM without pyridoxine but with kinetin and 2,4D (Expts. 1 & 11). Malt extract inhibited callus formation (Expt. 3) which was reversed by pantothenate (Expt. 5). Callus formation also took place with petioles on RWBM containing 2,4D and kinetin and CH or GA_3 and vitamin C (Expts. 14 & 17).

(b) Growth of teak callus on subculture

For the survival of callus on its first subculture 14 different combinations of media were tried (Table 6). WBM with 2,4D, CM, pantothenate and biotin supported slight growth of callus (Expt. 1). RWBM supplemented with vitamin C, 2,4D, kinetin and CH gave better growth of callus than without vitamin C (Expts. 8 &6) although it was a good callus forming medium with primary explants. Optimum growth of callus on its first subculture was obtained on Knop's basal medium (KBM) having 2,4D, kinetin and CH (Expt. 13). Coconut milk was superior to kinetin (Expts. 14 and 15), while biotin and pantothenate showed an inhibitory effect (Expts. 13 & 14). KBM with 2,4D, kinetin and CH did not support the growth of the callus on its

Table - 6

Survival of teak callus on subculture

Basal medium : WBM, RWBM, KBM, $MSBM_1$

Other supplements (ppm) : NAA - 1; 2,4D - 0.6; IAA - 5; Kinetin - 1; CH - 200; pantothenate - 0.1; biotin - 0.1; vit.C - 0.1; tryptophan - 1; caffeine - 1; GA_3 - 10; inositol - 10; CM - 15%.

Period of incubation : 35 to 40 days. Results - as in Table 5. NT: Not tested

No.	Basal medium	Auxin	Cytokinin	Other supplements	Sucrose	Results of subculture 1	2	3
1	WBM	2,4D	CM	pantothenate, biotin	2%	+	NT	
2	WBM+Pyridoxine	2,4D	Kin	pantothenate, biotin, CH, vit.C	2%	+	"	
3	RWBM+Pyridoxine	2,4D	CM	CH, vit.C	"	++	"	
4	"	2,4D	CM	CH. pantothenate, biotin	"	++	"	
5	"	2,4D	Kin	CH, pantothenate, biotin	"	++	"	
6	RWBM	"	"	CH	"	++	"	
7	"	"	"	Vit.C	"	++		
8	"	"	"	CH, Vit.C	"	+++	++	NT
9	"	"	CM	YE, ME	"	NT	+++	"
10	"	NAA	CM	CH, Vit.C, GA_3	"	NT	++	"
11	"	IAA	Kin	CH, Vit.C, GA_3	"	"	NT Light brown +++0.4 g/T	

Table 6 contd.

12	RWBM	NAA	CM	CH	2%	NT	NT	Dark brown +++ 0.6 g/T	
13	KBM	2,4D	Kin	CH	2%	++++	+	NT	
14	"	"	"	CH,pantothenate,biotin	2%	+	NT	NT	
15	"	"	CM	CH, Vit.C,tryptophan	"	+	NT	"	
16	"	"	"	CH,Vit.C,tryptophan	"	+	!	"	
17	"	NAA	"	CH,vit.C,tryptophan,GA_3	"	++	"	"	
18	"	2,4D	"	YE, ME	"	++	"	"	
19	"	2,4D 6 ppm	"	CH,vit.C,tryptophan,inositol	"	NT	+	"	
20	"	" "	Kin	CH, tryptophan	"	"	-	"	
21	"	" 0.6	"	CH,tryptophan	"	"	+	"	
22	"	" 6 ppm	"	CH,tryptophan,caffeine	"	"	-	"	
23	"	" "	"	CH (200 ppm)	"	"	+	"	
24	"	" "	0.5 ppm	CH, tryptophan	"	NT	+	"	4th sub-culture
25	"	" 6 ppm	CM 10%	CH, tryptophan	2%	"	++	"	NT
26	"	" "	CM	""	"	"	++	"	"
27	"	" ""	-	CH,Vit.C,tryptophan,caffeine, inositol	"	"	+	"	"
28	"	" "	-	CH,Vit.C,GA_3,inositol	"	"	+	"	"
29	"	NAA	CM	CH,Vit.C, GA3	"	"	++	"	"

Table 6 contd.

30	KBM	2,4D	CM	YE, ME	3%	NT	++	NT	NT
31	$MSBM_1$	IAA	Kin	CH	3%	NT	++++	Yellow compact 01260 mg	++++ 01068 mg
32	"	"	"	CH,vit.C,GA_3	"	"	++++	Bright yellow 01080mg	NT
33	"	NAA	"	"	"	"	++	NT	NT
34	"	IAA	"	YE,ME	"	"	+++	"	"
35	"	2,4D	"	YE,ME	"	"	++	"	"
36	"	IAA	CM	CH	"	"	NT	"	Bright yellow 1880 mg
37	"	"	Kin.	CH, GA_3	"	"	"	"	Yellow 01400 mg
38	"	"	"	CH, vit.C	"	"	"	"	Yellow 867 mg

second subculture (Expt. 13). Similarly, KBM with CH, vitamin C, caffeine, inositol tryptophan, gibberellic acid, NAA 2,4D, YE and ME in various combinations (Expts. 17-30) was found to give poor growth of callus. When the basal medium was changed to Murashige and Skoog's ($MSBM_1^2$) and supplemented with IAA, kinetin and CH best callus growth occurred (Expt. 31). It must be noted that $MSBM_1^2$ contained 1 ppm of glycine instead of 2 ppm. GA_3 and vitamin C (Expt. 32) under these conditions did not further enhance growth. When IAA was replaced by 2,4D (Expts. 34 and 35) or NAA (Expts. 32, and 33), growth was poor even in the presence of kinetin, indicating that IAA is the best auxin for the proliferation of this callus. Even in the 4th subculture it was noted that CM was better than kinetin (Expts. 31 & 36).

On medium 36 & 37 the colour of teak callus progressively changed from bright yellow to orange and the pigment gradually diffused into the medium. The isolation of the pigmented component in callus tissue was therefore attempted. The callus was maintained on $MSBM_1$ supplemented with IAA, CH and CM upto the 10th subculture. As described in the next chapter (Chapter III), after the 10th subculture CH was omitted and the level of glycine was increased to 3.2 ppm. After the 15th subculture the level of glycine was maintained at 2 ppm as in Murashige and Skoog's original report. (Plate 1)

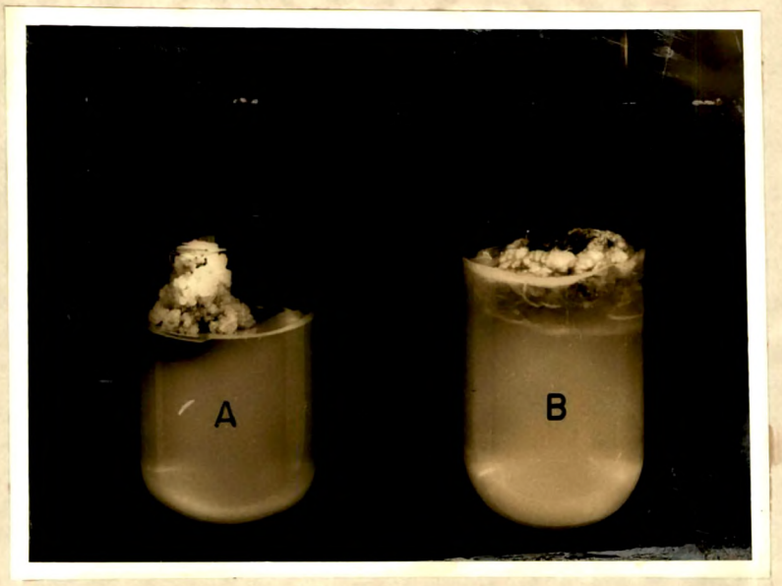


Plate I

(A) Initiation of teak callus on explant

(B) Teak callus

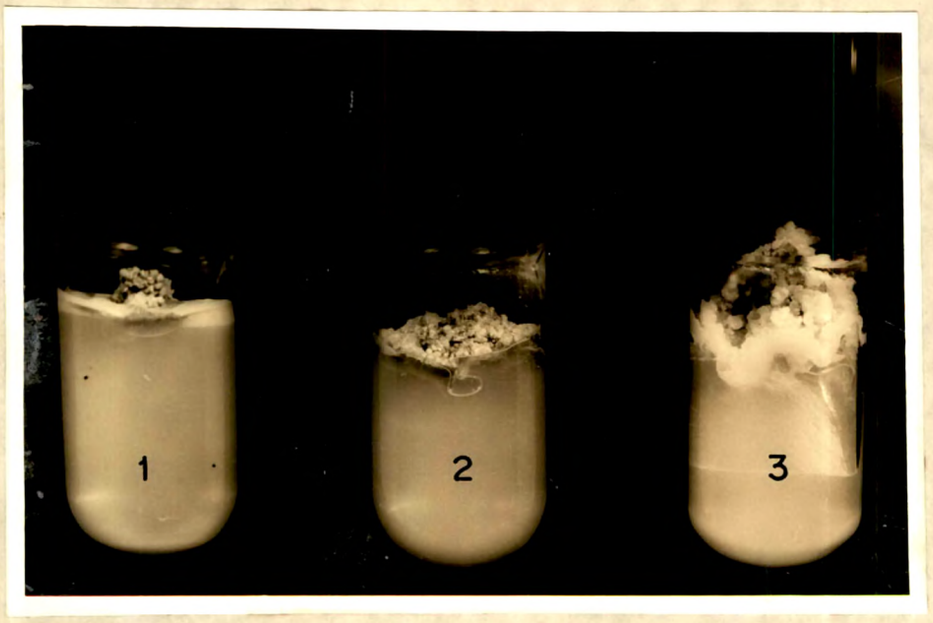


Plate II

Callus of (1) Jack; (2) Mulberry and (3) Poplar.

SECTION 2

a) Initiation of callus from jack, mulberry and poplar

The seeds of jack tree were obtained from a freshly cut fruit and sterilized as follows. They were successively washed with detergent, distilled water, 70% alcohol and 10% hypochlorite solution. Further manipulations were carried out asceptically. After 15 min of hypochlorite treatment the seeds were washed free of chlorine with sterile distilled water, transferred to sterile tubes having moist cotton and allowed to germinate at 28°C in the dark. After 10-15 days the seedlings were transferred to Petri dishes and the shoot regions cut into 2-4 mm long segments and inoculated into test tubes containing different media.

Explants of mulberry and poplar were obtained from small branches (1-2 cm diameter), sterilized and inoculated into media according to the method employed for teak.

Jack callus

Explants from seedlings were cultured on 30 different media (Table 7). Callus formation was initiated on WBM supplemented with various growth factors (Expts. 2-10 and 12), but not when 2,4D and kinetin (Expts. 11-13) were used together. When a pure sample of pigments normally present in the jack tree was added to the medium, callus formation was inhibited (Expts. 14 & 17). WBM having NAA, CM, YE, ME and inositol gave good callus formation but better callus formation was noticed when BTOA replaced NAA (Expts. 4 & 12). CM was better than kinetin for callus proliferation (Expts.12 & 14). At lower concentrations

Table - 7

Effect of different media on callus formation from jack

Basal medium : WBM, RWBM, SBM and KBM.

Sucrose : 2%

Results as in Table 5.

Other supplements (ppm) : NAA - 1; 2,4D - 0.6; BTCA - 1; YE - 1000; ME - 1000; kinetin - 1; inositol - 10; CM - 10%.

No.	Basal medium	Auxin	Cytokinin	Other supplements	Results
1	WBM	NAA	CM		No callus formation
2	"	"	"	YE	+
3	"	"	"	YE, ME	++
4	"	"	"	YE, ME, Inos	+++
5	"	2	"	YE,ME,Inositol,pantothenate	+++
6	"	"	"	Pantothenate	+
7	"	"	"	Pantothenate, biotin	+
8	"	"	"	Pantothenate,biotin,inositol	++
9	"	2,4D	"	Pantothenate,biotin,inositol	+
10	"	"	"	YE, ME, Inositol	+
11	"	"	Kin	YE, ME, Inositol	-

Table 7 contd.

12	WBM	BTOA	CM	YE, ME, Inositol	++++
13	"	2,4D	Kin	YE,Me,Inositol,glutamine	-
14	"	BTOA	"	YE,ME,inositol	+
15	"	"	"	YE,ME,Inositol,Artocarpin	-
16	"	"	"	YE,ME,Inositol,cycloartocarpin	-
17	"	"	"	YE,ME,Inositol,artocarpesin	-
18	"	"	"	YE,ME,Inositol 0.5 ppm	+
19	"	"	5%	YE,ME,Inositol	+
20	"	"	CM	YE 0.5%, ME 0.5%, Inositol	++
21	RWBM	2,4D	CM	YE, ME	++
22	"	"	Kin	YE,ME, glutamine	-
23	KBM	"	CM	YE,ME	-
24	"	"	Kin	YE,ME,glutamine	-
25	"	"	"	YE, ME	-
26	SBM	"	CM	YE, ME	-
27	"	"	"	YE, ME,Inositol	-
28	"	"	"	CH, Try.	-
29	"	"	"	CH,Try,Inositol,Caffeine	-
30	RWBM	"	Kin	YE, ME	-

of inositol (0.5 ppm) or CM (5%) or YE (0.05%) or ME (0.05%) there was very little or no callus formation (Expts. 18-20). There was no callus formation on any other basal medium except RWBM having 2,4D, CM, YE and ME (Expt. 21).

Mulberry callus

The optimum requirement for callus formation in mulberry (Table 8) was a basal medium (WBM, RWBM or $MSBM_1$) supplemented with pantothenate, biotin, 2,4D (IAA) and kinetin (or coconut milk) (Expts. 2,6 & 7). IAA and 2,4D together yielded better callus formation than NAA alone (Expt. 7 & 8). The initiation of callus was observed 10-15 days after inoculation and after 35 days the tissue was subcultured. The callus was white in colour when grown on (Expts. 2 & 6) WBM and RWBM and tan in colour on $MSBM_1$ (Expt. 7).

Poplar callus

The initiation of callus from primary explants of poplar was noticed 10-15 days after inoculation on media noted in Table 9. The minimum requirements for callus formation were NAA, CM or kinetin, pantothenate, biotin with $MSBM_1$ or RWBM. The best callus formation was observed with $MSBM_1$ containing NAA, kinetin, pantothenate, biotin and 3% sucrose.

b) Growth on subculture

The experimental results on the survival of callus of poplar, jack and mulberry on repeated subculture are given in Tables 9, 10, & 11 respectively. The best callus forming media for jack explants did not

Table - 8

Effect of different media on callus formation from mulberry

Basal medium : WBM, RWBM, $MSBM_1$

Other supplements (ppm) : 2,4D - 0.6; BTOA - 1: NAA - 1; Kinetin - 1; YE - 1000; ME - 1000; pantothenate - 1; biotin - 0.1; CM - 15%. Results as in Table 5

No.	Basal medium	Auxin	Cytokinin	Supplements	Sucrose	Results
1	WBM	2,4D	CM	YE, ME	2%	-
2	"	"	"	Pantothenate,biotin	%	+++ whitish callus
3	"	2,4D	"	CH,vit.C,inositol,GA3, caffeine	"	++
4	RWBM	2,4D	Kin	-	"	++
5	RWBM-Pyr	"	"	CH	"	-
6	RWBM	"	CM	Pantothenate,biotin	"	+++ Whitish callus
7	$MSBM_1$	IAA,2,4D	Kin	Pantothenate,biotin	3%	+++ dark colour
8	$MSBM_1$	NAA	Kin	Pantothenate,biotin	"	+
9	"	"	//	YE, ME	"	+

Table - 9

Effect of different media on callus formation and survival of poplar.

Basal medium : RWBM, $MSBM_1$, WBM, KBM, HBM.

Other supplements: (ppm) NAA - 1; Kin - 1; pantothenate - 0.1; biotin - 0.01, CM - 15%.

Results as in Table 5

No.	Basal medium	Auxin	Cytokinin	Supplements	Sucrose	Callus initiation	Sub-culture		
							Ist	2nd	3rd.
1	RWBM	NAA	Kin	Pantothenate & biotin	2%	++			+ 290 mg
2	RWBM	NAA	CM	"	2%	+++			
3	$MSBM_1$	NAA	Kin	"	3%	++++	++++	++++	++++ 1382 mg
4	KBM	"	"	"	2%				+ 360 mg
5	WBM	"	"	"	2%				+ 270 mg
6	HBM	"	"	"	2%				+ 235 mg

Contain Murashige & Skoog's vitamin and glycine (Table 3C) solution instead of original solution reported.

support growth on subculture, but when supplemented with artocarpine, a pigment from jack wood (Expts. 2 & 3; Table 10) a little growth was observed when tissues were incubated at 26°C. The effect of artocarpine was not observed when callus was grown at 29-30° (Expt 4&5) a temperature found suitable for jack callus. Further studies on this compound were not pursued. Since $MSBM_1$ was found suitable for the cultivation of teak callus, this basal solution with BTOA, CM, YE and ME was also tested and found to be good for the survival of jack callus. Either BTOA and CM or kinetin and IAA were equally effective as supplements (Table 10; Expts. 7 and 8), whereas relatively poor growth was observed with BTOA and kinetin (Table 10, Expt. 9). To eliminate the possibilities of the presence of unknown factors especially phenolics in CM, Blaydes medium with 2,4D and kinetin was tested. This medium with or without 10 ppm of tyrosine and phenylalanine singly or together gave good growth (Table 10; Expts. 11 & 12).

Since mulberry resembled teak in showing better callus formation on a high salt medium it was transferred to RWBM or $MSBM_1$ with pantothenate, biotin, YE, ME, CM or kinetin, IAA or 2,4D, in different combinations (Table 11). 2,4D with RWBM (Expt. 2) or $MSBM_1$ (Expt. 6) as well as NAA (expt. 5) gave poor growth. YE and ME singly or pantothenate and biotin together gave good growth (Expts. 4, 7 & 8). Since the medium (Expt. 4) is chemically defined the callus has been maintained routinely on this medium by subculture after every 35 days. (Plate - II

Table - 10

Survival of jack callus on subculture

Basal medium : WBM, MSBM, BBM.

Other supplements (ppm): NAA - 1; IAA - 5; BTOA - 1; YE - 1000; ME - 1000; Inositol - 10 ppm
CH - 200; phenylalanine - 1; tyrosine - 1; kinetin - 1; CM - 15%.

Results as in Table 5

No.	Basal medium	Auxin	Cytokinin	Other supplements	Sucrose	Temp. °C	Ist sub	2nd sub	3rd sub	4th sub	9th sub
1	WBM	NAA	CM	YE, ME, Inositol	2%	24-26	-				
2	WBM	BTOA	"	YE,ME,Inositol	"	"	-				
3	"	"	"	" + Artocarpine	"	"	+				
4	"	"	"	YE, ME, Inositol	"	29-30	+				
5	"	"	"	" + Artocarpine	"	"	+				
6	$MSBM_1$	"	"	YE,ME,Artocarpine	3%	"	+++	+++	+++	+++	
7	"	"	"	YE, ME	"	""	+++	+++	+++	++++	++++
8	"	IAA	Kin	YE, ME	"	"	NT	NT	NT	+++	
9	"	BTOA	Kin	YE, ME	"	"	"	"	"	++	
10	"	"	CM	CH	"	"	"	"	"	++	
11	BBM	2,4D	Kin(0.5)	-	2%	"	"	"	"	"	+++++
12	"	"	"	Phenylalanine tyrosine	"	"	"	"	"	"	+++++

Table - 11

Survival of Mulberry callus on subculture

Basal medium : RWBM, $MSBN_1$

Other supplements: 2,4D - 0.6; IAA - 5; NAA - 1; Kinetin - 1; pantothenate - 0.1; biotin - 0.1; YE - 1000; ME - 1000; CM - 15%.

Results as in Table 5

No.	Basal medium	Auxin	Cytokinin	Supplements	Sucrose	Ist sub-culture	2nd sub-culture	3rd sub-culture	4th sub-culture
1	RWBM	2,4D	CM	Pantothenate, biotin	2%	++	++	NT	NT
2	"	2,4D	Kin	"	"	++	NT	"	"
3	"	IAA	"	"	3%	NT	NT	+++	"
4	$MSBN_1$	IAA	"	"	"	+++	++++	++++	++++
5	"	NAA	"	"	"	+	NT	NT	NT
6	"	2,4D	"	"	"	NT	+++	"	"
7	"	IAA	"	YE	"	"	"	++++	"
8	"	IAA	"	ME	"	"	"	++++	"
9	"	2,4D 6 ppm	"	Pantothenate, biotin	"	"	"	+++	"

The different media tried for survival of poplar callus are listed in Table 9. In the case of poplar the best medium for callus induction is also the best for survival on subculture. When different basal media (Expts. 3-6) were tried it was noted that MSBM with NAA, kinetin, pantothenate and biotin gave optimum growth. The culture is routinely being maintained on this medium and subcultured every 25-30 days.

SECTION 3

Change of growth rate of the cultures on subculture

The growth rate of the cultures which was very low initially increased with subculture (Figs. 4 & 5). The growth rate of teak showed marked fluctuation. With jack callus (Fig.5) maintained on Blayde's medium, the growth rate was more uniform throughout. A steady increase in growth was observed with mulberry cultures after the ninth subculture (Fig. 5). The rate of growth changed very markedly with poplar callus. In the first few subcultures the growth ratio (ratio of final fresh weight to initial inoculum weight) was 10-15 whereas later it was 30-40.

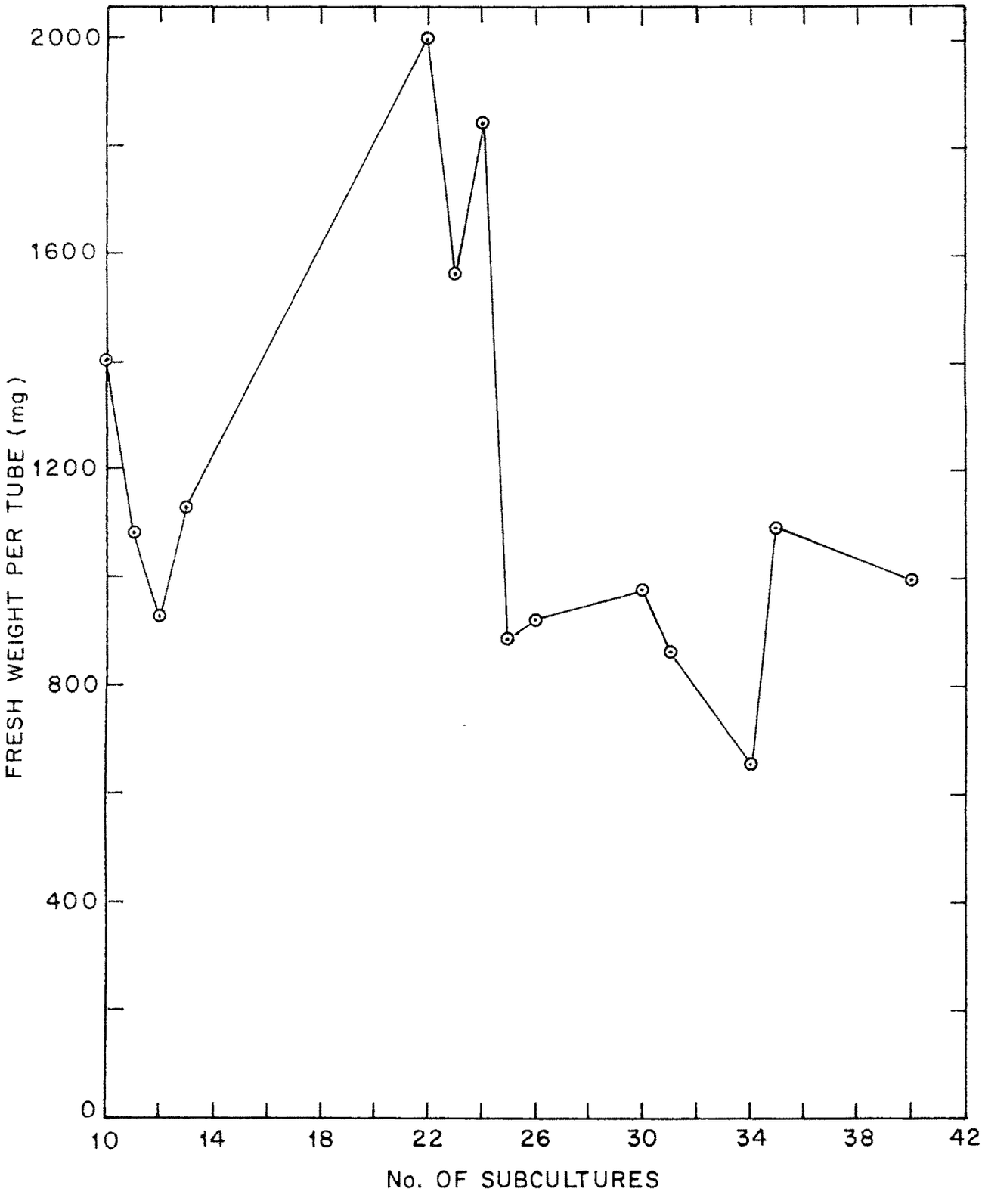


FIG. 4 GROWTH OF TEAK CALLUS ON SUBCULTURE

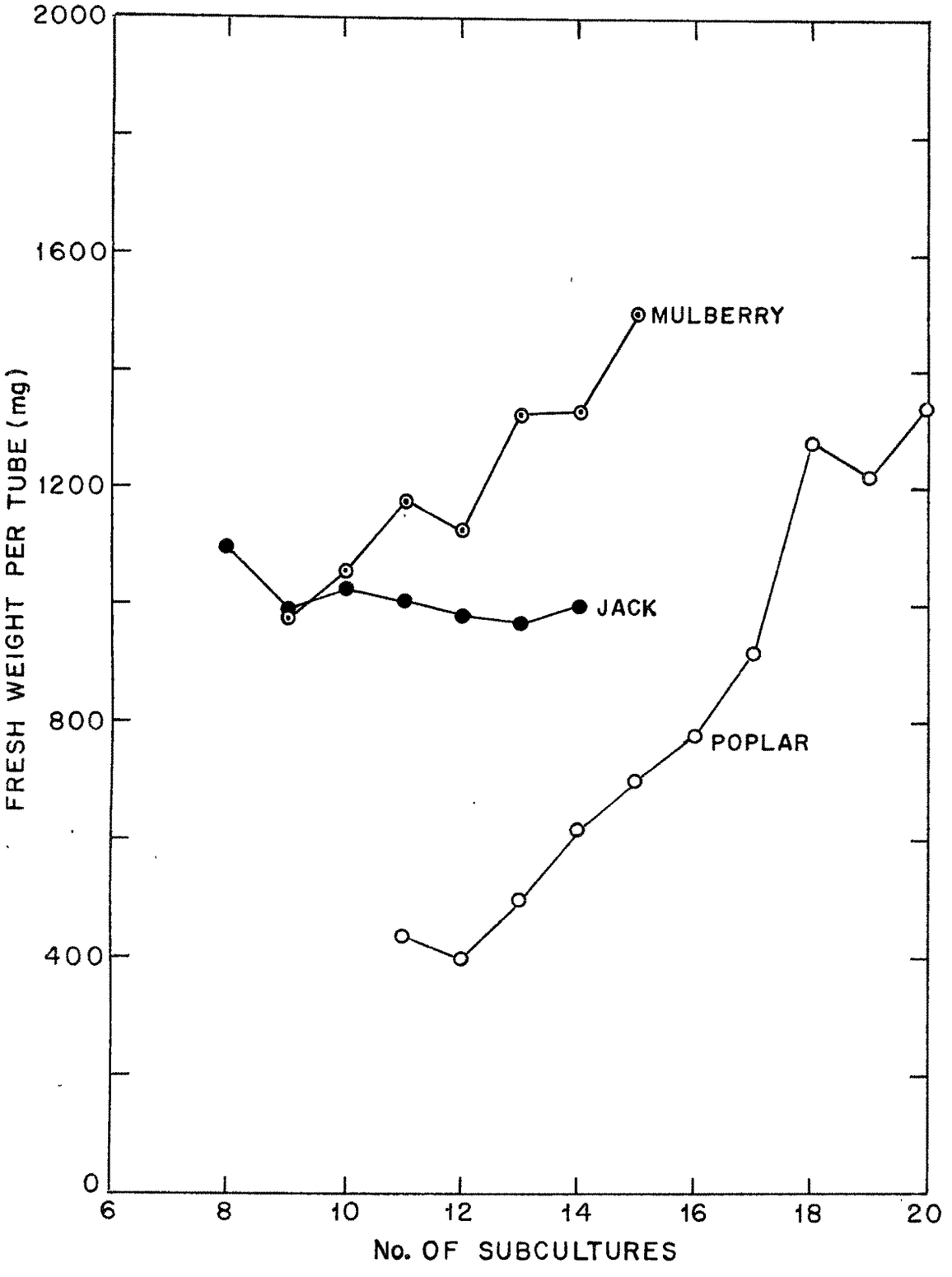


FIG. 5 GROWTH OF JACK, MULBERRY & POPLAR ON SUBCULTURE

SECTION 4

a) Appearance of the callus

Teak, poplar and jack grown on agar medium formed undifferentiated masses of cells. Teak callus was compact, hard and light yellowish-orange to brown in colour depending on the composition of the medium and incubation period. After 20-25 days the callus gradually sank into the semisolid medium. When callus was grown on $MSBM_1$ supplemented with CH, IAA and kinetin it was pale yellow in colour after 25-30 days. On the other hand by addition of GA_3 or by replacement of kinetin with coconut milk the callus was bright yellow to orange in colour. The callus turned brown after 40 days in medium containing CM, and after 60 days in medium having kinetin. Callus of jack and poplar was soft and friable. Jack callus was brown and poplar callus white, till the 25th subculture, but later slowly turned pale yellow and at present is light brown. Mulberry callus consisted of a mass of brown undifferentiated cells with a few white roots. These roots in general did not grow beyond 1-2 mm and were not affected by the length of the incubation period, except in a few cases where some roots thickened and grew to a length of 2-3 cm.

b) Reproducibility of the Results

Tissue cultures were established from the branch segments of teak in four independent experiments using RWBM supplemented with 2,4D, kinetin and CH or RWBM (without pyridoxine) with 2,4D, CM, CH and vitamin C, from mulberry in two experiments using RWBM with 2,4D, CM pantothenate and biotin or $MSBM_1$ with IAA, 2,4D, kinetin, panthothenate

and biotin; from poplar in two experiments using MSB_1 with NAA, kinetin, pantothenate and biotin and from jack seedlings in three experiments using WBM with BTOA, CM, YE, ME and inositol. Callus initiation was visible after 25 days with jack, whereas with the other three trees it took about 15 days.

All the cultures were viable on their respective media for more than six years and with the exception of jack they are being routinely maintained in this laboratory.

The results of these experiments indicate that viable cultures can be obtained from branch segments of teak, mulberry and poplar and from seedlings of jack. For jack and teak the requirement for callus formation is a basal medium supplemented with auxin, cytokinin, CH, YE or ME. Mulberry and poplar formed good callus when the basal medium was supplemented with an auxin, coconut milk, pantothenate and biotin. Kinetin could replace coconut milk for poplar callus formation. Mulberry callus could be maintained on $MSBM_1$ supplemented with IAA, kinetin, pantothenate and biotin, which differed from callus initiating media by the absence of 2,4D. The media required for callus formation and maintenance were the same for poplar (i.e. $MSBM_1$ with NAA, kinetin, pantothenate and biotin). With teak and jack the media for callus initiation and maintenance were different and a basal medium containing a high salt concentration gave good growth.

SECTION 5

Discussion

The primary purpose of the present study was to obtain viable callus cultures from some of the forest trees on a chemically defined medium, and this objective has been attained for teak, jack, mulberry and poplar.

The minimum requirements for the formation of callus by all the four tree explants were a basal medium containing mineral salts, vitamins, glycine, an auxin and kinetin or coconut milk. Supplements like pantothenate and biotin were found to enhance callus initiation from explants of teak (Table 5, expt. 7), mulberry (Table 8, Expt. 2) and poplar (Table 9, Expts. 1, 2 & 3) but not from jack (Table 7, Expt. 7). The mineral salt composition of the basal medium influenced callus formation in the case of poplar (Table 9, Expts. 1 to 3), but not of mulberry and teak. Various workers have shown that different tissues differ in their auxin requirements. Although it is not certain whether an auxin is necessary for callus formation since the explants in the present study were not grown on auxin free media, the results with different auxins show differences in callus formation (Table 7, Expts. 8 & 9 and 4 & 12) and (Table 8, Expts. 7 & 8). Media without kinetin or coconut milk were tested only for teak explants and the results definitely indicate that for callus proliferation these growth factors are necessary (Table 5, Expts. 21 &11). Coconut milk was found to be growth enhancing for teak (Table 5, expts. 19 & 20), jack (Table 7, Expts. 12 & 14) and poplar (Table 9, Expts.1&2).

Malt extract was found inhibitory for teak (Table 5, Expts. 2 & 3) and stimulatory for jack (Table 7, Expts. 2 & 3). The enhancement of growth by inositol with jack tissue (Table 7, Expts. 3 & 4) may be due to the "antinecrosis effect" mentioned by Jacquiot (1964).

Although teak and jack formed callus on RWBM or WBM with different supplements, they did not survive on subculture on these media (Table 6, Expt. 6 and Table 10, Expts. 1 & 2). Hence different media were tried for the survival of these tissues. Teak callus easily proliferated on $MSBM_1$, medium supplemented with IAA, kinetin and CH (Table 6, Expt. 31) and growth was better than on RWBM supplemented either with 2,4D, kinetin and CH (Table 6, Expt. 6) or with CH, IAA and CH (Table 6, Expt. 12). Similar results were observed with jack where $MSBM_1$ was found to be better for survival than WBM (Table 10, Expts. 4 & 7). The only difference between the callus initiation and callus maintenance media for mulberry was the elimination of 2,4D from $MSBM_1$ containing pantothenate, biotin, kinetin, IAA and 2,4D (Table 11, Expt. 4). Mulberry grew better on $MSBM_1$ than onRWBM (Table 11, Expts. 3 & 4). Poplar callus could be maintained indefinitely on the same medium used for initiation (Table 9, Expt. 3). It also grew better on a high salt containing medium (Table 9, Expts. 3 to 6). These results support earlier reports (Murashige and Skoog 1962 and Borchert 1967) that added phytohormones and other growth factors can exert better effect if supplied at optimum salt concentrations. IAA was the best auxin for teak (Table 6, Expts. 32 & 33 and 34 & 35), whereas jack grew better with BTOA (Table10, Expts. 8 & 9). Coconut milk was clearly superior to kinetin for teak (Table 6, Expts. 31 & 36).

Jack had a specific temperature requirement of 30°C and the tissue did not survive when incubated at a lower temperature (26°C). The other tissues grew well over the temperature range of 26-30°C.

One of the objectives of growing callus tissues from the four trees was to devise a chemically defined medium for their growth. Teak callus could grow on $MSBM_1$ containing kinetin and IAA (Chapter III). Jack could be grown on Blaydes' basal medium containing 2,4D, kinetin, phenylalanine and tyrosine (Table 10, Expts. 11 and 12). Mulberry was grown on $MSBM_1$ supplemented with IAA, kinetin, pantothenate and biotin (Table 11, Expt. 4) while poplar survived on $MSBM_1$ supplemented with NAA, kinetin, pantothenate and biotin (Table 9, Expt. 3). All these are synthetic media of known composition.

In most of the reports on the cultures of woody trees the tissue died on subculture and only in a very few cases have viable cultures been obtained . The tissues isolated from explants of teak, jack, mulberry and poplar in the present studies have been maintained by repeated subculture without any diminution in growth for over six years and can be considered as viable cell lines of these four trees. These are the first reports of viable callus cultures from these four trees.

C H A P T E R - III

NUTRITIONAL REQUIREMENTS OF TEAK CALLUS CULTURE

***

SECTION 1

The nutritional requirements of teak callus were studied in detail after obtaining viable callus as described in the previous chapter. The effect of different mineral salt compositions, nitrogen and carbon sources, auxins, cytokinins, gibberellins, growth retardants and other supplements, pH and temperature were studied. In some cases the optimum concentration of some of the nutrients was determined. The experimental conditions regarding the inoculum, medium, period of growth etc. are given in Chapter I. It should be noted that the objective of the present study was not merely to define the conditions for obtaining good growth of the callus but also good pigmentation. Rapid growth of colourless tissue with no pigmentation would be of no value for the study of the phenolic pigments. The experiments described in this chapter are not in chronological order. The variation in the final weights of the tissues grown on the same medium may be due to the period for which the cultures had been maintained in vitro when the experiment was performed. The growth of teak tissue varied from one subculture to the other as described in Chapter II. Although the results in each Table can be compared, the results in two different within Tables under identical conditions are not necessarily comparable. Hence controls were run in each experiment and the significance of each experiment has been discussed with reference to the control in each case. Carry over of nutrients with the inoculum could also have influenced some of the results. The effect of kinetin clearly demonstrates this point as the tissue did not grow when subcultured

repeatedly on a medium without kinetin, although it grew well in the first subculture.

In most of the Tables used for the growth of teak callus, the changes in the composition of the media are given with reference to the medium (Chapter 1). Both wet and dry weights were determined and were taken as a measure of growth. The standard error of mean has been discussed earlier in Chapter I. On the basis of these results a particular effect was arbitrarily considered as significant only when the growth obtained differed from the control by at least 15 to 20 per cent.

(a) Mineral nutrition

Several inorganic solutions have been used for the cultivation of plant tissues in vitro. Of these, six different basal inorganic salt solutions, differing either in the concentration or number of salts supplied, were used for the growth of teak callus. No change was made in the original composition of inorganic solutions as reported by the respective workers. The micronutrient requirements were not studied. The organic supplements were supplied according to Murashige and Skoog's (1962)medium.

On a dry and wet weight basis teak tissue grew best on Murashige and Skoog's medium (Table 12). Although on its first subculture the callus grew on Smith's (1967) as well as Murashige and Skoog's (1962) media, on subsequent subcultures the wet and dry weights were markedly reduced on the former. Teak callus did not survive on Heller's (Gautheret 1959) or Reinert and White's

Table - 12

Effect of different basal media

Medium: Mineral salts of different composition
Organic supplement - Murashige & Skoog's (Table 3C); glycine 1 ppm
IAA - 5 ppm; kinetin - 1 ppm; sucrose 3%.

No.	Subculture number / Media	1		2		3	
		Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight (mg)
1	White's	402	40	322	31	301	30
2	Heller's	242	25	148	27	-	-
3	Smith's	836	80	388	35	418	50
4	Reinert & White	132	15	172	23	-	-
5	Blaydes'	255	28	346	33	601	58
6	Murashige and Skoog	731	75	890	842	872	85

(Wolter and Skoog, 1966) mineral solutions on subculture. On White's (1954) medium, the callus gradually diminished in weight, while on Blaydes' (1966) medium it gradually increased in weight.

The colour of the callus was also different with different mineral salt compositions. On White's minerals it was pale yellow, on Heller's brownish yellow, on Smith's very light yellow and on Reinert and White's blackish brown in colour. The appearance of callus on Blaydes' and Murashige and Skoog's solutions was similar in being compact and coloured yellow to orange brown on Murashige and Skoog's medium and orange yellow on Blaydes' medium. In all cases the callus grew deep into the media. Subsequent work was carried out on Murashige and Skoog's medium as it gave optimum growth with good pigmentation.

(b) Nitrogen

The main source of nitrogen in Murashige and Skoog's medium is ammonium and potassium nitrate. The effect of different nitrogen sources is shown in Table 13. Potassium was supplied in equivalent amount as the chloride and nitrogen was added either in an inorganic or organic form. All the organic compounds except urea and allantoin were added at concentrations given in the Table and not in amounts equivalent to the nitrogen present in Murashige and Skoog's medium. Optimum growth occurred when standard Murashige and Skoog's medium was used. Fairly good growth was observed with potassium or ammonium nitrate and yeast extract while ammonium sulphate, ammonium chloride and calcium and sodium nitrate did not

Table - 13

Effect of different nitrogen sources

Media: Mineral salts - Murashige and Skoog's without any nitrogen source
Organic supplements - Murashige and Skoog's (Table 3C) + glycine 1 ppm.
IAA - 5 ppm; kinetin - 1 ppm; sucrose - 3%
Nitrogen sources added at 0.840 g N/1000 ml medium

No.	Subculture number / Nitrogen sources	1		2		3	
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
1	KNO_3 + NH_4NO_3(MSB medium)	1048	101	816	75	872	82
2	KNO_3	865	77	1070	130	506	60
3	NH_4NO_3	684	59	768	67	546	49
4	$Ca(NO_3)_2$	237	28	294	40	-	-
5	$NaNO_3$	229	15	256	40	176	28
6	$(NH_4)_2SO_4$	222	29	113	13	-	-
7	NH_4Cl	149	18	125	13	-	-
8	Urea	399	48	209	24	204	14
9	Allantoin	765	85	629	73	388	35
10	Casein hydrolysate-400 ppm	286	48	324	50	188	35
11	Coconut milk 15%	270	47	209	43	156	24
12	Yeast extract 0.2%	752	110	735	111	613	87
13	Malt extract 0.2%	322	56	134	24	80	16

give good growth. Poor growth was observed with all the organic nitrogen sources except allantoin and yeast extract. Growth on allantoin decreased on subculture. All the organic nitrogen sources gave higher dry weight of the tissue compared to the standard medium. The moisture content of the tissue was variable and was least with yeast extract.

Nitrogen sources influenced the pigmentation and appearance of callus. Calcium and sodium nitrate and ammonium sulphate and chloride gave a black or brownish black, hard compact tissue. On potassium nitrate the callus was yellow to black and imparted a red colour to the medium. A light yellow, soft, spreading callus was observed with ammonium nitrate and allantoin although the colour was lighter with the latter. On coconut milk, casein hydrolysate and malt extract the callus was black in colour, on urea it was pale yellow and soft while on yeast extract it was yellowish brown and compact.

(c) Carbon sources

The effect of different carbon sources which included different carbohydrates was tested on teak tissue (Table 14). Sucrose was omitted and the carbon sources were added at 3% concentration. All the media contained inositol at 100 ppm. Teak callus grew best on sucrose and glucose on its first subculture. The results with galactose were striking. There was an increase of almost 700 per cent in the final wet weight of tissue with galactose in its second subculture compared to the first and this effect was maintained in its third subculture also. A similar but much lower

Table - 14

Effect of different carbohydrate sources

Medium: Murashige & Skoog's mineral salts (Tables 3A & 3B)
Murashige & Skoog's organic supplements (Table 3C)
Kinetin - 1 ppm; IAA - 5 ppm; glycine 1 ppm
Carbohydrates at 3% concentration

No.	Subculture number / Carbohydrates	1		2		3		(mg)
		wet wt.	dry wt.	wet wt.	dry wt.	wet wt.	dry wt.	
1	Sucrose	827	85	980	111	815	79	
2	Glucose	720	70	2068	185	1222	97	
3	Fructose	300	25	922	99	828	56	
4	Maltose	637	36	798	39	1098	65	
5	Galactose	374	35	2757	271	2388	240	
6	Xylose	202	10	205	14	309	24	
7	Starch	176	15	70	5	-	-	
8	Sorbose	72	12	-	-	-	-	

Table - 15

Effect of different concentrations of sucrose on teak callus

Medium: Murashige & Skoog's mineral & organic solutions (3A, 3B & 3C)
Other supplements: IAA - 5 ppm; kinetin - 1 ppm; glycine 1 ppm.
GA_3 - 10 ppm

No.	Subculture number / Sucrose concentration (%)	1		2		3	
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt. (mg)
1	No sucrose	136	9	36	2	-	-
2	1	256	21	476	36	1081	88
3	2	368	30	1236	101	1523	125
4	3	862	83	893	98	925	90
5	4	823	71	2022	205	1100	110
6	5	1086	87	272	25	305	28

increase was also observed on subculture with fructose and glucose. Starch and sorbose did not support growth while xylose gave negligible growth.

On sucrose the callus grew as a compact, yellow to dark yellow coloured cell mass; with glucose a white soft callus was observed and with fructose it was a black compact mass. On maltose the tissue grew as a soft pale yellow spreading culture. With galactose the tissue grew as hard yellowish callus.

Sucrose Concentration

The effect of different concentrations of sucrose was tested on teak tissue (Table 15). On its first subculture 3 to 5 per cent sucrose gave better growth than at lower concentration, the water content with 5% sucrose being high. On subculture the growth was very poor with 5% sucrose whereas there was marked increase in growth with 1 to 2 per cent sugar. In the third subculture the growth with 3 and 4 per cent was as good as with 1 per cent sucrose.

(d) Amino Acids

As indicated in Chapter II, teak callus was grown on media having casein hydrolysate. In this experiment casein hydrolysate was omitted and the amino acids, grouped as shown in Table 16 were added to the $MSBM_1$ medium (containing 1 ppm glycine) at concentrations equivalent to those present in 200 ppm of casein hydrolysate. From the data it can be observed that glycine alone could give optimum growth of callus. Growth in the presence of methionine, threonine, DL-valine and DL-isoleucine or with media having all the amino acids

Table - 16

Effect of different amino acids

Media: Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements (ppm) - Niacin - 0.5; pyridoxine - 0.5; thiamine - 0.1; glycine - 1; meso-inositol - 100.

Other supplements - Kinetin - 1; IAA - 5; sucrose - 3%.
Amino acids added at concentration equivalent to those present in casein hydrolysate - 200 ppm.

No.	Amino acids supply	Wet weight	Dry weight (mg)
1	Arg + Lys + Met * Gly	319	38
2	Glu + Tyr + Thre	328	34
3	DL Asp + His + DL Val	322	38
4	DL Leu + Phenyl alanine + DL Isoleu	203	34
5	Arg + Glu + DL Asp + DL Leu	288	33
6	Lys + Tyr + His + Phenyl alanine	267	31
7	Met + Thre + DL Val + DL Isoleu	470	50
8	Arg + Asp + Glu + Gly + His + Isoleu + Leu + Lys + Met + Phenylala + Thr + Tyr + Val	489	50
9	Glycine	526	59
10	Casein hydrolysate	423	50

Table - 17

Effect of glycine, gibberellic acid & nucleotide bases

Media - Murashige & Skoog's mineral salts (Tables 3A, 3B)
Organic supplements - Murashige & Skoog's (glycine - 1 ppm) (Table 3C)
Other supplements (ppm) - kinetin - 1; IAA - 5; casein hydrolysate - 200; glycine - 2.2; gibberellic acid - 100; nucleotide bases - 2 (each of adenosine) cytidylic acid, uracil, guanidine and thymidine HCl, sucrose 3%.

No.	Subculture number / Media supplements	1		2		3	
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
		mg					
1	Casein hydrolysate	493	49	1397	135	710	80
2	Casein hydrolysate + gibberellic acid	489	48	1294	115	1618	153
3	Glycine	529	55	1079	95	1199	120
4	Glycine + GA_3	575	57	1744	171	1710	166
5	Casein hydrolysate + Nucleotide bases	521	54	1421	140	758	91
6	Casein hydrolysate +GA_3+Nucleotide bases	555	56	1433	-	1382	135
7	Glycine + Nucleotide bases	812	70	1281	115	940	103
8	Glycine + Nucleotide bases + GA_3	368	71	1100	190	1476	138

Table - 18

Glycine concentration

Media. Mineral solution Murashige & Skoog's (Tables 3A & 3B)

Organic supplements - Vitamins Murashige and Skoog's (No glycine added, Table 3C)
Kinetin 1 ppm; IAA 5 ppm; sucrose 3%

No.	Subculture number 1 Glycine conc. ppm.	Wet wt.	Dry wt. mg
1	0.0	972	192
2	2.0	1268	184
3	3.0	1044	120
4	5.0	1176	208
5	10.0	1328	200

as present in casein hydrolysate was the value as that obtained with casein hydrolysate.

The effect of casein hydrolysate, gibberellic acid, nucleotide bases and an increased glycine level were tested with Murashige and Skoog's medium containing 1 ppm glycine and the results are given in Table 17. From the data it can be observed that an increased glycine level gave better growth than casein hydrolysate especially in the third subculture. When gibberellin and nucleotide bases were added alone or combined to the medium a little increase in growth was observed. Optimum growth was however obtained with glycine and gibberellic acid. From the above results it can be concluded that glycine alone can replace casein hydrolysate. The effect of glycine concentration is shown in Table 18 and it can be observed that glycine concentration of 2-10 ppm was equally effective. For further work 2 ppm of glycine was used instead of 1 ppm.

(e) Effect of different vitamins

Murashige and Skoog's medium contains thiamine, nicotinic acid, pyridoxine and inositol only. The effect of these vitamins was determined by testing the effect of the individual vitamins and mixtures of vitamins. From Table 19 it can be seen that when thiamine, pyridoxine and nicotinic acid were eliminated from the medium the growth of the callus was poor but significant. The callus grew very well with thiamine alone on its first subculture,

Table - 19

Effect of different vitamins on teak callus

Media: Mineral salts - Murashige and Skoog's (Tables 3A & 3B)

Organic supplements: Inositol - 100 (ppm), glycine - 2; sucrose - 3%; kinetin - 1; IAA - 5.

No.	Subculture number / Vitamins	1 Wet wt.	1 Dry wt.	2 Wet wt.	2 Dry wt.	3 Wet wt.	3 Dry wt. (mg)
1	No vitamins	356	25	553	49	533	42
2	Thiamine 0.1 ppm	954	109	751	60	643	46
3	Nicotinic acid 0.5 ppm	487	58	703	57	554	72
4	Pyridoxine HCl 0.5 ppm	459	59	671	60	1103	142
5	Thia + Nic.acid	869	98	726	60	514	52
6	Pyr + Nic.acid	345	39	788	71	490	56
7	Pyr + Thia	2453	200	1172	120	886	88
8	Thia + Nic.+Pyr.	1000	109	1066	98	950	92

Table - 20

Effect of inositol and glycine on callus growth

Media: Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements: IAA - 5 ppm; kinetin - 1 ppm; sucrose 3%

No.	Subculture number / Supplements	1		2		3	
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt. (mg)
1	MA + IAA + Kinetin	188	20	115	13	73	7
2	+ glycine 2 ppm + IAA + Kinetin	148	16	102	12	126	14
3	+ Inositol 100 ppm + IAA + Ki	854	87	856	83	730	80
4	+ glycine 2 ppm + inositol 100 ppm	812	78	881	84	647	65
5	+ glycine 2 ppm + inositol 100 ppm + Nic.acid 0.5 ppm + Thia 0.1 ppm + Pyr. 0.5 ppm	1292	105	1319	117	1285	110

Table - 21

Effect of different concentrations of inositol on teak callus

Media: Mineral salt - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements(ppm) Murashige & Skoog's (Table 3C)
Glycine 1 ppm; IAA - 5; kinetin - 1; sucrose 3%

No.	Subculture number	1		2		3	(mg)
	Concentration of inositol (ppm)	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
1	0.0	126	17	803	7.0	787	6
2	25.0	959	75	823	65.0	750	52
3	50.0	1402	100	1028	80.0	536	45
4	75.0	1376	98	1018	76.0	562	38
5	100.0	1521	130	1686	120.0	1118	80
6	150.0	1551	110	494	38.0	688	52
7	250.0	1060	76	1170	80.0	976	73

but on subsequent subcultures the growth was negligible. Nicotinic acid showed some stimulation whereas pyridoxine showed a three fold increase in growth on the third subculture. Of the three combinations of two vitamins each (Expt. 5,6,7) only pyridoxine and thiamine showed marked stimulation especially with first two subcultures. Maximum growth was observed when all the three vitamins were added.

Table 20 shows the effect of inositol and glycine which were not omitted in the previous experiment. As the callus could grow without pyridoxine, thiamine and nicotinic acid, in this experiment all the three vitamins were omitted. It can be observed from the results that teak callus slowly died on subculture when inositol and glycine were omitted. The callus barely survives when glycine was added at 2 ppm. Inositol at 100 ppm, increases the growth of callus strikingly in all the subcultures. Glycine and inositol together did not increase the growth more than inositol alone. The best growth was obtained on media containing all the organic supplements of Murashige and Skoog's medium.

Concentration of inositol

Inositol was generally added at 100 ppm. As inositol was shown to be essential for the growth of teak callus, its optimum concentration was determined. As noted above, callus died on subculture in the absence of inositol. The optimum concentration of inositol was found to be about 100 ppm (Table 21).

(f) Auxins

The effect of different auxins was tested by omission of IAA, which was generally added at 5 ppm and addition of the auxins

Table - 22

Effect of different auxins on teak callus

Mineral salts: Murashige & Skoog's (Tables 3A & 3B)
Vitamins : As per Murashige & Skoog's medium (Table 3C)
Other supplements (ppm): Glycine - 1; CH - 200; Kinetin - 1; IAA - 5; sucrose - 3%.

No.	Subculture number / Media	1 Wet wt.	1 Dry wt.	2 Wet wt.	2 Dry wt.	3 Wet wt.	3 Dry wt.
					mg		
1	Standard medium without IAA & Kin	90	9	-	-	-	-
2	1 + IAA	178	18	300	30	348	35
3	1 + Kinetin	123	12	66	7	-	-
4	1 + Kinetin + IAA	1081	110	932	90	1128	114
5	3 + IBA 5 ppm	278	30	112	12	108	11
6	3 + IPA 5 ppm	762	75	440	45	380	35
7	3 + α-NAA 2 ppm	1147	115	524	50	680	70
8	3 + β -NAA 2 ppm	688	70	340	35	362	32
9	3 + 2,4D 2 ppm	158	16	79	8	-	-
10	3 + 2,4,5 T 2 ppm	188	20	67	7	-	-
11	3 + GA_3	156	15	55	6	-	-

Table - 23

Effect of different concentrations of IAA on teak callus

Mineral salts - Murashige & Skoog's medium (Tables 3A & 3B)

Vitamins - Murashige & Skoog's medium (Table 3C)
Glycine, inositol and kinetin, GA_3 - 10 ppm; sucrose 3%

No.	Subcutre number / IAA concentration(ppm)	1		2		3		(mg)
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	
1	0.0	98	7	75	6	60	5	
2	0.1	1321	94	1012	78	1210	82	
3	1.0	1598	126	1284	96	1432	130	
4	5.0	1790	179	1820	178	1920	186	
5	10.0	1958	143	1968	140	1834	130	

at the concentrations shown in the Table 22. IAA was found to be the best auxin for the growth of the callus. The callus did not proliferate in the absence of auxin and cytokinin. On subculture slight growth was maintained even in the total absence of kinetin, but not in the absence of auxin. IBA, α-NAA, IPA and β-NAA supported growth, each compound being more effective than the preceding one, but none of them gave as good growth as IAA. 2,4D and 2,4,5-T did not support the growth of the callus.

The callus was pale yellow to orange when grown on IAA, whitish yellow with α-NAA, necrosis set in with 2,4-D and 2,4, 5-T while IBA and IPA gave dark brown coloured callus.

The effect of different concentrations of IAA is shown in Table 23. Optimum growth occurs at 5 ppm IAA. Auxin is essential for the growth of teak callus.

(g) Cytokinins

Callus tissue of teak was grown on media having different cytokinins (Table 24). Teak callus could grow on media without cytokinin but at a slow rate. Coconut milk gave maximum growth of callus. Of the chemically defined compounds kinetin gave maximum growth. With diphenyl urea and zeatin there was a gradual growth stimulation on subculture. Growth with thiourea was less than with other compounds.

The callus turned very pale yellow without any cytokinin. With zeatin and DPU it was more yellow. Maximum pigmentation was visually observed in coconut milk containing media though kinetin also gave bright yellow callus.

Table - 24

Effect of different (cytokinins)

Media: Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements - Murashige & Skoog's (Table 3C) glycine 1 ppm.
IAA - 5 ppm; sucrose 3%

No.	Subculture number / Cytokinins	1		2		3		
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	(mg)
1	No cytokinin	231	23	171	17	379	38	
2	Kinetin 1 ppm	1233	123	1212	124	1099	100	
3	CM 15%	1783	183	1897	145	1850	181	
4	Diphenylurea 1 ppm	340	37	504	53	863	82	
5	Thiourea 0.2 ppm	194	20	1004	91	569	50	
6	Zeatin 0.1 (γ/6)	324	11	594	60	915	80	

Table - 25

Effect of different concentrations of kinetin on teak callus

Mineral salts - Murashige & Skoog's (Tables 3A & 3B)

Organic supplements - Murashige and Skoog's (Table 3C), glycine - 1 ppm, IAA - 5 ppm; sucrose - 3%; GA_3 - 10 ppm.

No.	Subculture number / Kinetin concentration (ppm)	1 Wet wt.	1 Dry wt.	2 Wet wt.	2 Dry wt.	3 Wet wt.	3 Dry wt.
							mg
1	0.0	305	28	260	25	392	35
2	0.1	1222	91	1023	110	989	100
3	0.5	1317	97	1223	107	1282	120
4	1.0	2106	202	1568	140	1862	175
5	2.0	1987	140	1626	135	1788	133
6	5.0	1855	120	1320	110	1520	90

The effect of different concentrations of kinetin on teak callus growth was noted (Table 25). Optimum growth was obtained at about 1 ppm. The callus survived even at 5.0 ppm kinetin.

(h) Gibberellins

Gibberellins, particularly gibberellic acid (GA_3) have been reported to have growth promoting activity in plants. As GA_3 at 10 ppm concentration was found to enhance the growth of teak callus in its initial stages, the effect of various gibberellins added aseptically to the medium was studied (Table 26). The weights obtained in these experiments show that there wasn no significant increase in the growth of the callus with any of the gibberellins, especially on subculture. The slight increase obtained with GA_3, GA_5 and GA_{13} requires confirmation. There was marked inhibition with GA_7.

There was no visual difference regarding pigmentation or type of growth of the callus with any of the compounds.However, when gibberellic acid was added to the medium and autoclaved, marked pigmentation of the tissue was observed (Chapter V).

Concentration of gibberellic acid

Table 27 shows the effect of different concentrations of GA_3. It can be seen that on subculture, as the GA_3 concentration increased tissue growth was reduced. At higher concentration of GA_3 50 ppm the tissue turned brown.

(i) Nucleic acid bases

The five nucleotide derivatives (adenine, guanine, cytidylic acid, thymidine and uracil) were added separately and together to

Table - 26

Effect of different gibberellins

Media: Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements - Vitamins & Glycine as per Murashige & Skoog's
IAA - 5 ppm; kinetin - 1 ppm and sucrose - 3%.
Gibberellins were added aseptically at 10 ppm.

	Subculture number	1		2		3	
No.	Gibberellins	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt. (mg)
1	Standard medium	857	83	954	88	997	93
2	GA_1	646	82	798	48	648	74
3	GA_3	897	102	946	96	858	101
4	GA_4	626	71	325	29	410	48
5	GA_5	754	92	654	91	952	112
6	GA_7	397	34	496	45	370	29
7	GA_4/GA_7	459	60	394	40	514	61
8	GA_9	590	68	496	51	621	56
9	GA_{13}	782	100	933	80	1042	106

Table - 27

Effect of different concentrations of gibberellic acid

Medium: Murashige and Skoog's medium (As per Table 26)

Aseptically
GA_3 added

No.	Subculture number / GA_3 concentration (ppm)	1		2		3	
		Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
1	0.0	2544	172	2250	125	2081	198
2	1.0	2456	163	2068	110	1980	176
3	5.0	2430	163	2108	115	1823	185
4	10.0	2113	140	1989	128	1680	170
5	20.0	1961	140	1884	119	1532	110
6	50.0	2169	154	1895	123	1564	115

the standard teak medium at a concentration of 2 ppm (Table 28). The results indicate that on the third subculture adenine and uracil were markedly growth enhancing whereas cytidylic acid was inhibitory. All the compounds together were not more effective than adenine or uracil alone. The callus in all the cases had an orange yellow colour and a compact appearance.

(j) Growth retardants

The effect of different growth retardants was studied on teak callus. It can be observed from Table 29 that at as low a concentration as 0.005 - 0.01 ppm, all the three growth retarding substances, ABA, CCC and AMO1618 reduced the growth of teak callus markedly. ABA and AMO 1618 at 1 ppm were strongly inhibitory, the growth being only 15-20% of the control and with CCC about 40%.

The appearance of the callus did not change when growth retardants were added, except that at higher concentrations necrosis was observed.

(k) GA_3 and ABA effect

ABA at four different concentrations was added under aseptic conditions to standard teak medium (Table 30). It was observed that ABA was increasingly inhibitory as the concentration was increased. When GA_3 was added to medium having ABA, it partially reversed the inhibition due to ABA.

In all the cases the callus was hard, compact and yellow to brown in colour. The colour became more brown when the concentration of GA_3 and ABA was increased.

Table - 28

Effect of nucleic acid bases

Media: Murashige & Skoog's mineral solution (Tables 3A & 3B)
Organic supplements - Vitamins and glycine - Murashige & Skoog's (Table 3C)
Kinetin - 1 ppm, Nucleic acid bases added
IAA 5 ppm; glycine 1 ppm; sucrose 3%.

No.	Subculture number / Media	1 Wet wt.	1 Dry wt.	2 Wet wt.	2 Dry wt.	3 Wet wt.	3 Dry wt. (mg/mg)
1	Standard medium	1062	80	816	73	930	86
2	Adenine 2 ppm	1064	77	797	66	1920	138
3	Cytydylic acid 2 ppm	1430	92	369	35	680	88
4	Uracil 2 ppm	1345	88	969	76	1740	141
5	Thymidine 2 ppm	1438	94	351	31	1256	64
6	Guanine 2 ppm	980	82	899	68	980	75
7	AD + Cy + U + Thy + Gua	1297	91	1148	100	1154	110

Table - 29

Effect of ABA, CCC & AMO 1618

Media: Mineral salts - Murashige and Skoog's (Tables 3A & 3B)

Organic supplements(ppm) - Murashige & Skoog's (Table 3C)

glycine-1ppm, IAA - 5.0 ppm; kinetin - 1 ppm; sucrose - 3%

No.	Supplements		Wet wt. (mg)	Dry wt.
1	Basal medium		1004	96
2	ABA	0.005	346	37
3	ABA	0.01	410	45
4	ABA	0.1	423	38
5	ABA	1.0	130	14
6	CCC	0.005	523	55
7	CCC	0.01	547	59
8	CCC	0.01	384	41
9	CCC	1.0	236	36
10	AMO 1618	0.005	655	85
11	AMO 1618	0.01	574	62
12	AMO 1618	0.1	519	56
13	AMO 1618	1.0	189	20

Table - 30

Effect of GA_3 and ABA

Media:
Mineral salts - Murashige and Skoog's (Tables 3A & 3B)
Organic supplements - Murashige & Skoog's (Table 3C), glycine - 1 ppm
Kinetin - 1 ppm; IAA - 5 ppm & sucrose 3%
ABA & GA_3 added asptically, concentrations in ppm.

No.	Supplements (ppm)		Wet wt. mg.	Dry wt.
1	-		1204	119
2	ABA	0.01	704	77
3	ABA	0.1	274	31
4	ABA	1.0	290	33
5	ABA	2.0	171	20
6	GA_3 1 + ABA	.01	1099	110
7	" "	.1	595	63
8	" "	1.0	586	54
9	" "	2.0	274	38
10	GA_3 10 + ABA	0.01	703	34
11	" + "	0.1	516	53
12	" "	1.0	656	65
13	" "	2.0	458	40
14	GA_3 50 + ABA	0.01	606	67
15	" "	0.1	443	44
16	" "	1.0	393	40
17	" "	1.0	492	48

(l) Temperature

Since growth of jack callus was found to be enhanced at higher temperature, teak callus grown on media with or without GA_3 was incubated at four different temperatures and the growth was measured (Table 31). It was found that teak callus grew best at about 30 ± 1°C. GA_3 had no measurable stimulatory effect at 26° - 30°C and at 40 days it was inhibitory at these temperatures at which there was good growth without GA. Teak callus generally was incubated at 27 ± 2°.

(m) Hydrogen ion concentration

In all the experiments on the effect of different growth factors, the pH of the medium was adjusted to 5.6 to 6. To find the optimum pH (Table 32) for the growth of teak tissue, the callus was grown on media at pH 4 to 8. On wet weight basis the growth of the teak callus obtained from pH 4 to 6 was about the same. At pH 7 the growth declined, and again at pH 8 it increased. The reason for this is not known. These results require confirmation. The change in pH during growth was not measured.

(n) Inoculum size

Table 33 shows the effect of inoculum size. The results of these experiments are represented by final weight and by the ratio of the final wet weight to the inoculum wet weight. With inocula of 30 - 135 mg of fresh tissue the ratio was nearly the same. Further work has to be done to establish whether the

Table - 31

Effect of temperature

Inoculum - 80 ± 20 mg.

No.	Temperature °C	MSB (Table 4-1) wet wt. (mg)		MSB + GA_3 10 ppm wet wt. (mg)	
		20 days	40 days	20 days	40 days
1	26 - 27	415	927	652	753
2	30 - 31	1367	1706	1348	881
3	37 - 39	290	256	410	349
4	44 - 45	261	173	161	153

Table - 32

Effect of pH

Medium:
Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements - Murashige & Skoog's (Table 3C)
IAA - 5 ppm; kinetin - 1 ppm & Sucrose 3%

pH	Wet wt. (mg)	Dry wt.(mg)
4	800	70
5	799	80
6	732	74
7	296	20
8	549	62

Table - 33

Effect of size of inoculum

Medium: Mineral salts - Murashige & Skoog's (Tables 3A & 3B)
Organic supplements - Vitamins & Glycine at the concentration of MSB medium (Table -1C), glycine-1ppm
IAA - 5 ppm; kinetin - 1 ppm; sucrose 3%

No.	Wt. of inoculum (mg)	Final average wt. (mg)	Ratio of final wt. / inoculum wt. (mg)
1	30	328	11
2	50	1188	24
3	115	1263	11
4	135	1391	10.4
5	250	1449	6.0

Table - 34

Different Agar Concentration

Medium: Minerals - Murashige & Skoog's (Tables 3A & 3B)

Organic supplements - Murashige & Skoog's vitamins and glycine(Table 3C)
glycine-1ppm; IAA - 5 ppm; kinetin - 1 ppm; sucrose 3%

Inoculum - 100 ± 20 mg

No.	Agar %	Wet weight	Dru weight
		mg	
1	0.5	738	68
2	0.7	932	138
3	0.9	242	102
4	1.1	83	86
5	1.3	796	84

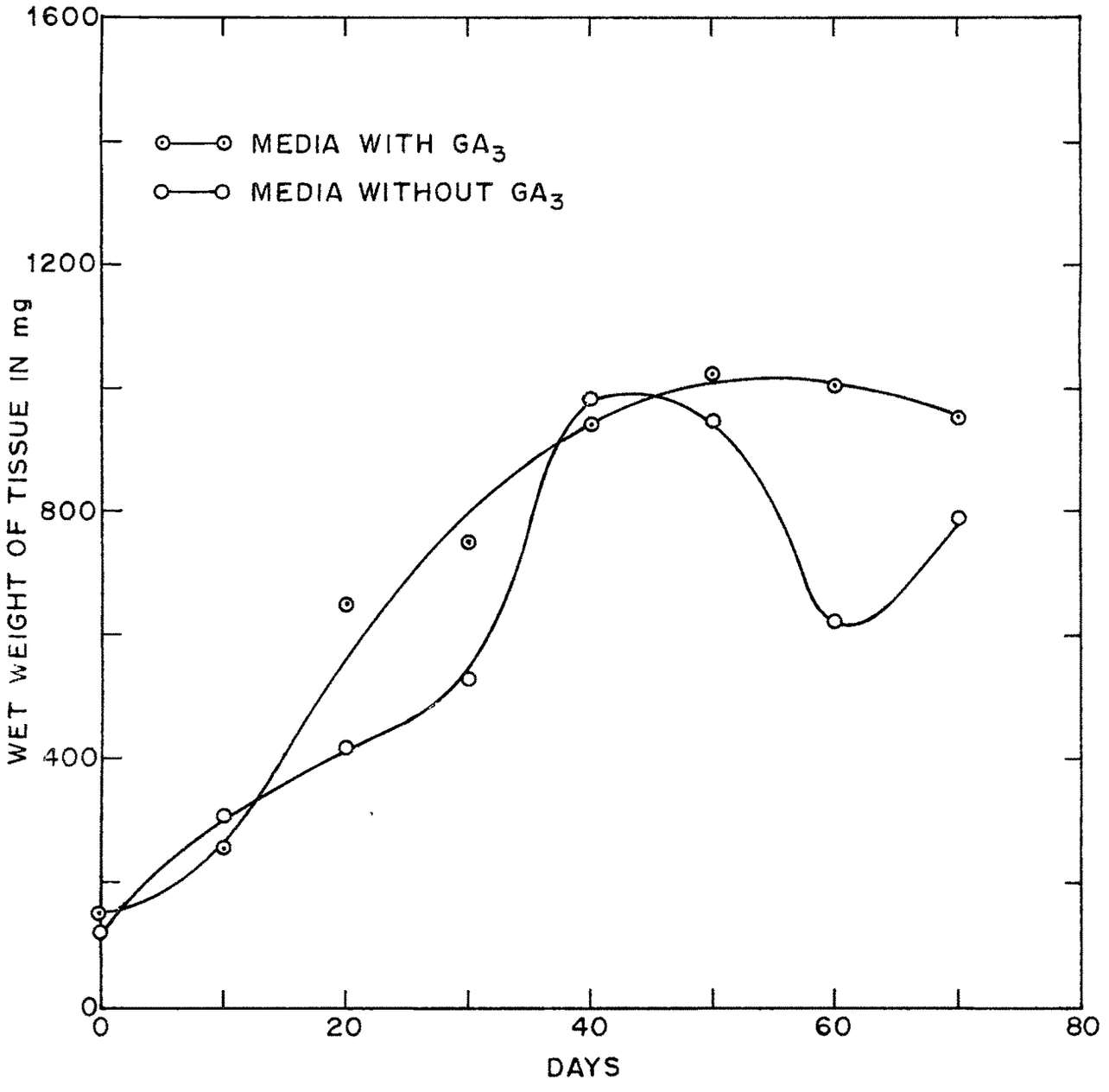


FIG. 6 GROWTH OF TEAK TISSUE

higher final ratio obtained with 50 mg inoculum size is of significance. The minimum inoculum size was not determined. The results of this experiment show that the inoculum generally used was sufficient to permit growth and that variation of size within definite limits did not affect the experiments provided that inocula in each set were nearly the same.

(o) Agar concentration

Agar is added to the medium as a semi-solid base for the growing tissue. At a low concentration of agar the medium is softer and the tissue gets submerged whereas at a higher concentration it affects the diffusion of nutrients to the tissue. Teak tissue grew best at 0.7% agar concentration(Table 34). With more than 0.9% concentration of agar teak tissue showed diminution of growth.

(p) Growth curve

Many of the subsequent experiments on the production of pigment were carried out with or without GA_3 in the medium. Hence growth curves were determined for both media.

The growth curve of teak callus with or without gibberellic acid (10 ppm) is shown in Fig. 6. There was no significant difference between the growth curves in the two media. There is a brief lag period followed by a period of almost linear increase in weight with time.

SECTION 2

DISCUSSION

It should be noted that in the present work the effect of several different factors was studied during three subcultures under the same conditions. This reduces the effect of carryover of nutrients with inoculum which can be of significance in many cases.

Mineral composition

The mineral composition of the medium has a profound effect on the growth of plant tissue and organ cultures. The inorganic salt solutions now being used for the cultivation of plant tissues *in vitro* have been developed by modifying the solutions of White (1954) which was originally used for root and later for callus cultivation and of Gautheret (1937).

Burkholder and Nickell (1949), Heller (1953), Hildebrandt, Riker and Duggar (1946) and Murashige and Skoog (1962) have demonstrated that the concentration of salts in White's and Gautheret's solutions are sub-optimal for some tissues. Nitsch and Nitsch (1956), Wood and Braun (1961) and Murashige and Skoog (1962) have shown that enhanced levels of nitrogen, potassium and phosphorus promoted the growth of a number of tissues. For the cultivation of tree callus *in vitro*, Jacquiot (1964) used Gautheret's mineral solution, Reinert and White (1956) modified White's solution by increasing the salt concentration, Mathes(1967)

used White's mineral solution while some workers (Gautheret 1959) used Gautheret's or Heller's mineral solution. This indicates that callus cultures from various trees have different salt requirements.

The optimum growth of teak callus on Murashige and Skoog's medium shows that the higher concentration of salts in the medium is beneficial. Successful cultures of Acer pennsylvanica and A. saccharum (Mathes 1967), Cupressus lusitanica (Borchert 1967) ash, pine, and oak (Wolter 1964), olive (Lavee and Messer 1969) were obtained when grown on media having high salt concentrations.

The failure of teak tissue to survive on White's, Heller's, and Reinert and White's media can be attributed to their lower nitrogen, phosphorous and potassium levels, but the growth on Smith's mineral salts is not easily explainable as this medium also has a low salt concentration. On Blaydes' medium which has a lower nitrogen and higher phosphate than Murashige and Skoog's medium, good growth was obtained. Though Murashige and Skoog's salt solution is adequate for the growth of teak callus it would require a large number of experiments to determine the optimum concentration of each of the macro elements. The micro elements present in each medium may also affect the growth of callus and its survival but these have not been studied.

Nitrogen sources

Riker and Gutsche (1948), Nickell and Burkholder (1950) and Heller (1953) reported that nitrogen was required as nitrate and

that their callus cultures could not utilize nitrite or ammonium salts. However, tomato root cultures at neutral pH could utilize ammonium as the nitrogen source (Hannay, Fletcher and Street 1959). Very few tree calli have been studied for their nitrogen requirement. Simpkin et al. (1970) reported that nitrate was essential for A. pseudoplatanus cells and growth was enhanced by urea, CH or a mixture of amino acids. Mango leaf gall callus (Jain and Arya 1966) grew well when a pancreatic digest of casein was used along with White's mineral salts. Steinhart et al.(1961) showed that ammonium nitrate was not useful for the cultivation of Picea abies callus culture, while White and Gilbey (1966) have shown that spruce callus could grow well on media supplemented with glutamine and ammonium nitrate, chloride or sulphate though on sodium nitrate growth was poor. De Torok and Thimann (1961) have shown that for their strain of spruce callus, asparagine was a better nitrogen source.

Teak callus grew best when supplied with nitrogen in the form of ammonium nitrate and potassium nitrate. Ammonium as sulphate or chloride, and nitrate as the sodium or calcium salt gave poor growth. The failure of these salts to give good growth may be due to the high concentrations of calcium, sodium, sulphate or chloride ions which had to be added to supply the required nitrogen level thereby upsetting the balance of ions in the medium.

Complex organic nitrogen sources such as casein hydrolysate, edamin, peptone, malt extract etc. have been used

extensively in studies of many plant tissue cultures (Gautheret 1955). Yeast extract was first used by White (1934) for the cultivation of tomato roots. Later it has been used for pollen cultures of Ginkgo biloba (Tulecke 1957) seed cultures of Santalum album (Rao 1965) and maize endosperm culture (Straus and LaRue 1954). Malt extract has been successfully used for the cultivation of Pinus strobus (Lowenberg and Skoog 1952), Picea abies (Steinhart et al. 1961), Vigna catjang (Mascarenhas, 1965). Casein hydrolysate has been utilized for the cultivation of maize endosperm cultures(Straus and LaRue 1954), mango leaf crown gall cultures (Jain and Arya 1966) and Thuja orientalis (Rao and Mehta 1969) callus culture. Edamin together with other inorganic nitrogen sources was found to be growth enhancing for tobacco callus (Murashige and Skoog 1962).

Compared to the other organic nitrogen sources studied for the growth of teak callus, yeast extract at 0.2% concentration gave good growth.

The poor growth on complex nitrogen rich substances like casein hydrolysate, malt extract etc. can be explained as suggested by Street (1966) that the absorbed amino acids might not be deaminated or their deamination products may not yield ammonium at sites where they can be effectively used.

Carbohydrate sources

Gautheret (1941, 1945) was the first to study the carbohydrate requirements of callus cultures. Similar studies were made by several workers and it was concluded that most

cultures grew well when supplied with sucrose, glucose or fructose (Hildebrandt and Ricker 1949 & 1953; Nickell & Burkholder 1950; Henderson 1954). Galactose was ineffective with most tissues, but tumor tissues of Vinca rosea (Hildebrandt & Ricker 1949 & 1953), and normal tissue of Sequoia sempervirens (Ball 1955), Acer pennsylvanicum (Mathes 1967) and A. pseudoplatanus (Simpkins et al. 1970) could readily utilise it. Sequoia (Ball 1955) could utilize mannose which is also not readily utilizable by most of the tissues. Maltose was readily taken up by normal carrot tissue, corn endosperm tissue (Straus and LaRue 1970), and A. pennsylvanicum (Mathes 1967) and A. pseudoplatanus (Simpkins et al. 1970) and tumor tissue of Vinca rosea, Tagetes erecta, Chrysanthemum fructescens, Helianthus annus (Hildebrandt and Ricker 1949, 1953), and mango leaf gall callus (Jain and Arya 1966). A. pennsylvanicum (Mathes 1967) had the ability to utilize raffinose. Hildebrandt and Riker (1949, 1953) have reported the utilization of pectin, dextrin and soluble starch by various tissues. Soluble starch has been found to be utilized by virus tumor tissue of Rumex acetosa (Nickell and Burkholder 1950), corn endosperm tissue (Straus and LaRue 1954) and callus tissue of Rubus fruticosus (Karstens and de Meester 1960) Juniperus communis (Constabel 1963), A. pennsylvanicum (Mathes 1967) and A. pseudoplatanus (Simpkin et al. 1970). Glycerol was utilised by carrot (Gautheret 1948a). Callus cultures from Malus pumila and Malus robusta could utilize sorbitol as

sole carbon source (Chang and Taper 1972). Like Acer sp. teak callus also could use a variety of carbohydrates, particularly monosaccharides like galactose, glucose and fructose and disaccharides like sucrose and maltose. Galactose in particular is an excellent carbon source.

Vitamins

White (1954) first included yeast extract at 0.01% concentration in tomato root media and later found that thiamine and glycine present in yeast could replace this extract. It has generally been observed that like organ cultures, callus cultures also require vitamins. Pyridoxine was found to be essential for ash (Wolter and Skoog 1966) and niacin for spruce callus (Risser and White 1964), but in many cases there is no proof that B/vitamins are essential for growth. The requirement for thiamine by tobacco callus could be replaced by high levels of kinetin (0.5 to 1 ppm) and at this level of kinetin, synthesis of vitamin B_1 was activated (Digby and Skoog 1966). Thiamine has been added for the growth of various woody tissues, Juniperus virginiana, Picea abies, Pinus banksiana, P. strobus, Citrus limonum, Populus deltoides and Quercus alba (Gautheret 1959).

The results with teak showed that it has no absolute requirements for pyridoxine, thiamine or niacin. Of these three, pyridoxine alone and pyridoxine and thiamine together highly

stimulated growth in the presence of IAA, kinetin, inositol and glycine. Nicotinic acid alone or together with pyridoxine and thiamine did not stimulate the growth of callus showing that it was not essential for growth. Czosnowski (1952a) demonstrated that certain callus tissues were not stimulated by thiamine as they already contained this vitamin. Other workers (Czosnowski 1952b; Paris 1955) have shown that a number of callus cultures require thiamine. Pyridoxine has not been shown to be essential or to enhance the growth of callus in many cases (Street 1969).

Inositol has been found to enhance growth of many callus cultures, (Risser and White 1964; De Torok and Thimann 1961; Pollard, Shantz and Steward 1961; Linsmaier and Skoog 1965) and also to be an essential requirement in some cases like, Fraxinus pennsylvanica (Wolter and Skoog 1966). The growth of teak callus was totally dependent on inositol even when IAA and kinetin were supplied. The optimum concentration of inositol for growth was found to be 100 ppm.

Auxins

Auxins are known to control cell elongation and several other processes in plants. Indole acetic acid is the naturally occurring auxin. Many synthetic auxins have been described, some of which are more active than IAA and are used in the cultivation of plant tissue cultures (Street 1969, Gautheret 1955). Callus cultures of Citrus limonum, Crataegus monogyna (Gautheret 1959) and Pinus nigra (Bodgavonic 1968b) required NAA for growth.

Jacquiot (1964) described four types of cultures based on their auxin, cytokinin and other growth factor requirements and showed that some callus cultures (e.g. Populus tremula, Ulmus campestris, Tilia parvifolia and Prunus aviam) required auxin for their proliferation. White pine (Harvey 1967) and maritime pine (Lavauda 1970) required IAA and NAA for callus formation. 2,4D which could initiate callus formation could not support growth on subculture in white pine. Sandalwood seeds (Rao 1965) formed callus when grown on media having IAA and kinetin. Pollen of Thuja orientalis formed callus with 2 ppm 2,4D (Rao and Mehta 1969). Winton (1968) has shown that at low levels of 2,4D (0.04 ppm) rooting of aged callus of aspen occurred. (~~Simpkin et al. 1970~~) Cell suspension cultures of A. pseudoplatanus (Simpkin et al.1970) required 2,4D which could not be replaced by IAA,and NAA at high levels gave poor growth. Pinus nigra (Bodgavonic 1968b) required 2,4D initially for callus development, but later NAA was required. Nobecourt and Kofler (1945) used IAA for rose cultivation in vitro. IAA was better than 2,4D for olive callus (Lavee and Messer 1969). IAA or NAA was added for the formation of callus in Pinus sylvestris (Bodgavonic 1968a). Citron tissue (Schroeder and Spector 1957) was found to respond to a wider range of GA_3 concentration in the presence of exogenous IAA. Callus cultures of Betula varucosa, Castanea vesca and Salix carro were not stimulated by auxin (Gautheret 1959).

Teak callus could not survive without auxin and showed an absolute requirement for it. Like Pinus nigra (Bodgavonic 1968b) cultures teak callus could be initiated on 2,4D containing medium but later it required IAA for growth. NAA was not as good as IAA at the concentration supplied. Stimulation with IAA occurred even at as low a concentration as 0.1 ppm. IAA was the only phytohormone required by teak callus culture, its optimum concentration being 5-10 ppm.

Cytokinins

Coconut milk was first used by Overbeek, Conklin and Blakeslee (1941, 1942) for the growth of immature embryos of Datura. Since then it has been found to stimulate cell division in other cultured tissues and has been used extensively for the cultivation of many tissues in vitro (Gautheret 1955, Street 1969). Unlike callus cultures obtained from Eucalyptus gomphocephala A.P.C., E. camaldulensis, Dehnk and E. gunni Hook stems (Jacquiot 1964), pollen of Thuja orientalis (Rao and Mehta 1969) and Ginkgo biloba (Tulecke 1957) and embryos of Ginkgo biloba (Wang and Li 1966), teak tissue did not have an essential requirement for coconut milk. Like Acer pseudoplatanus (Henshaw et al. 1966), A. pennsylvanicum and A. saccharum (Mathes 1967) callus cultures which grew in CM and later on kinetin, teak callus also could grow on kinetin over repeated subculture. The growth of teak callus in CM containing medium was double that obtained on kinetin medium and was similar to

Cupressus cultures (Borchert 1967) which had a greater requirement for CM than kinetin.

Diphenyl urea was isolated from coconut milk by Shantz and Steward (1955) and shown to have cytokinin activity by Strong (1958). Bruce, Zwar and Kefford (1965) tested a number of substituted ureas and thioureas and found that most of them have cytokinin activity. Carew and Schwarting (1959) observed that DPU was able to support the growth of rye embryo callus when added with CH and 2,4D. Mascarenhas (1965) have reported the stimulatory effect of DPU on maize, rice, sorghum and wheat calli in the presence of high levels of NAA (5 ppm). Teak callus was stimulated only slightly by DPU and thiourea in comparison with kinetin.

The stimulatory effect of kinetin on the growth of tissue cultures has been extensively reviewed (Street 1969). Risser and White (1964) reported the depressing effect of kinetin at concentration from 10^{-8} to 10^{-6}M on spruce tissue cultures. Wolter and Skoog (1966) reported a stimulatory effect of kinetin in the presence of 2,4D. for Fraxinus, Pinus pinaster (Lavaud 1970) and sandalwood seed callus (Rao 1965) were formed in the presence of kinetin and an auxin. Pinus monticola (Harvey 1967) callus was stimulated by kinetin and P. nigra (Thomas 1972) hypocotyl tissue was cultivated in the presence of kinetin. Olive callus required kinetin after a number of passages on media without kinetin (Lavee and Messer 1969). At high levels of kinetin in an auxin free medium Acer pseudoplatanus cell suspensions formed

aggregates (Simpkins et al. 1970). In maple wood callus the extracellular hemicellulose formed in the presence of high kinetin had a reduced amount of galactose and xylose and an increased content of glucose (Simpkin and Street 1970). For the growth of teak callus kinetin was found to be stimulatory but not essential and the callus was sensitive to kinetin even at 0.1 ppm level in the presence of 5 ppm IAA. The pigmentation was more than with DPU, thiourea or zeatin, indicating that kinetin affects pigment metabolism of teak callus.

Zeatin at 0.1 r/l level enhanced growth of teak callus. Letham (1963) and Miller and Witham (1964) found that zeatin was much more active than kinetin in inducing cell division in carrot root and soyabean callus tissue cultures. Zeatin grown teak callus with this kinin was white indicating that cell metabolism/and kinetin is different.

Gibberellins

After the detection of gibberellins in higher plants (Paleg 1965; Lang 1970) a number of workers have tested their effect on the growth of cultured tissues. Netien (1957, 1958) and Nickell and Tulecke (1954) found that the tissues tested by them were either insensitive to or inhibited by gibberellin. Growth of callus tissue of Solanum tuberosus, Melilotus stem wound virus tumor (Street 1969) and Fraxinus pennsylvanicum (Wolter and Skoog 1966) was enhanced by gibberellin. For the continuous culture of tissues from staminate cones of Cupressus funebris gibberellin(GA_3) was found to be essential (Straus and Epp 1960). GA_3 in conjunction with IAA gave good growth of Nicotiana (Murashige 1964).

Teak callus growth was initially stimulated by GA_3, but later on the stimulatory effect was not observed (Tables 17 & 27). Recently many different gibberellins (Long 1970) have been isolated from different plants. None of the gibberellins tested had a stimulatory effect on teak callus but a marked inhibition was observed with GA_7.

Nucleotide Bases

Very few workers have reported the effect of nucleotide bases on tissue culture (Murashige and Skoog 1962). Callus formation in excised corn endosperm (Tamaoki and Ullstrup 1958) and growth of rye embryo callus (Carew and Schwarting 1958) and spruce callus (Risser and White 1964) was not affected by adenine. Tobacco tissues (Murashige and Skoog 1962) increased in weight by 50% when supplied with Braun's solution (CMP, GMP, L-asparagine and L-glutamine). Growth of maize tissue was enhanced by cytidylic acid and adenine and inhibited by uracil, wheat tissue was stimulated in the presence of all the nucleotides added, while rice tissue was not stimulated in studies carried out in this laboratory (unpublished). Teak callus showed inhibition of growth with cytidylic acid and enhancement with uracil and adenine.

Growth Retardants

The effect of growth retardants has been studied more in intact plants than in organ or callus cultures. Dolichos lablab (Tung and Raghavan 1968) root cultures were inhibited by CCC,

phosphon and B-995 at various concentrations and it was noted that the inhibition was due to a reduction in the frequency of cell division. Abscisic acid (ABA), CCC and AMO 1618 were found to be inhibitory for teak callus cultures at very low levels. Blumenfield and Gazit (1970) found that at low levels of kinetin 10 ppm ABA is inhibitory but the inhibition is overcome by increasing the kinetin level in the medium. Li, Rie, Rohrbaugh and Wender (1970) reported that at higher and lower concentrations, GA_3 overcomes ABA inhibition of growth and lignin synthesis and partially reverses ABA inhibition of scopolein production in tobacco tissue culture. Recently Altman and Goren (1971) reported the promotion of callus formation by ABA in the citrus bud. Although ABA inhibited growth of teak callus, GA_3 could reverse this inhibitory effect.

Temperature and Light

Most of the plant tissues are incubated at 23° to 33°C. The optimum temperatures for Spruce tissue was 20° (Risser and White 1964), for wheat cultures 25° (Shimada, Sasakama and Tsunewaki 1969) and for barley and wheat 27°C (Gamborg and Eveleigh 1968) respectively. Cultures in this laboratory are grown at 26° ± 1°C though teak callus grew best at 30° ± 1°C in darkness.

Teak callus showed very good growth under light and turned green. However, there was also a higher temperature in the light chamber. This effect was not studied further.

Hydrogen ion concentration

The optimum pH for sunflower and tobacco tissues was found to be between 4.5 to 6.0 for spruce between 5.5 to 6.5 (Risser and White 1964), for maize endosperm callus (Straus and La-Rue 1954) between 6.1 to 7 while normal and diseased Pennisetum typhoides gave good growth at pH 6.0 (Tiwari and Arya 1967). It was observed that at neutral pH, growth of teak was minimum and optimum growth was between pH 4 to 6.

Inoculum size

Plant cultures generally grow poorly if the inoculum size is too small. Growth of teak cultures was nearly the same over a wide range of inoculum weights (30-135 mg) though generally 100 ± 7 mg wet weight callus was used. The effect of very small inocula was not tested.

C H A P T E R - IV

QUINONES FROM TEAK CALLUS

***

SECTION 1

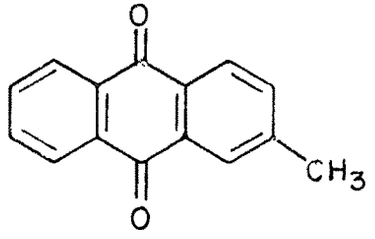
Experimental

One of the main purpose for growing callus tissues from the woody trees was to determine whether the phenolics normally present in the wood of these trees can also be synthesised by tissues in culture, and also to determine if any new phenolic compounds are produced by these cultures. The earlier chapters dealt with the initiation, maintenance, and nutritional requirements of the forest trees with particular reference to teak. This chapter describes the methods used for the extraction, isolation and characterisation of some compounds (anthraquinones) from callus tissue of teak. Fig. 7 shows anthraquinones obtained from teak heartwood (Sandermann and Simatupang, 1966).

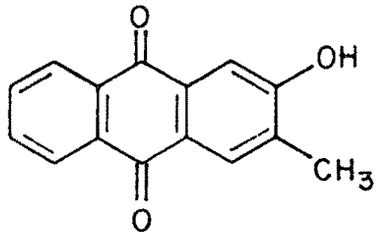
I Detection of anthraquinone in callus cultures

In preliminary experiments one tube of each of the callus cultures grown on two media (1,2; Table 2) was harvested and each callus extracted separately with cold and hot acetone. The yellow coloured extract was filtered, concentrated and spotted on silica gel plates (150 x 50 x 1 mm). The chromatogram was developed with different solvent systems, of which benzene containing 5% acetone was found to be best. All the extracts showed the presence of three yellow spots having Rf values very near to each other, and a fourth yellow spot moving with solvent front.

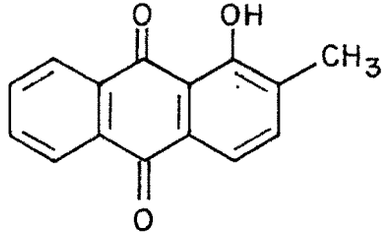
FIGURE-7



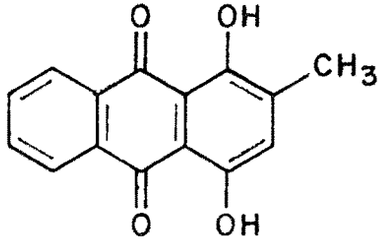
TECTOQUINONE



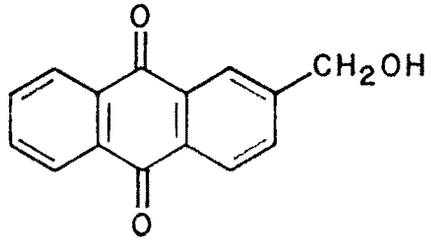
2-HYDROXY-3-METHYL
ANTHRAQUINONE



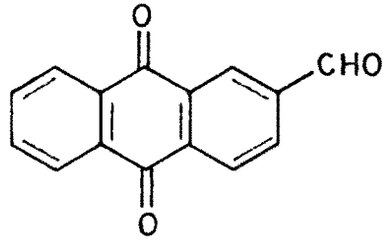
1-HYDROXY-2-METHYL
ANTHRAQUINONE



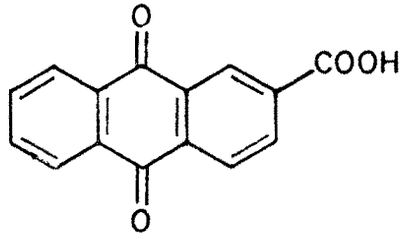
1,4-DIHYDROXY-2-METHYL
ANTHRAQUINONE



2-HYDROXYMETHYL-
ANTHRAQUINONE



ANTHRAQUINONE-2-ALDEHYDE



ANTHRAQUINONE-2-CARBOXYLIC ACID

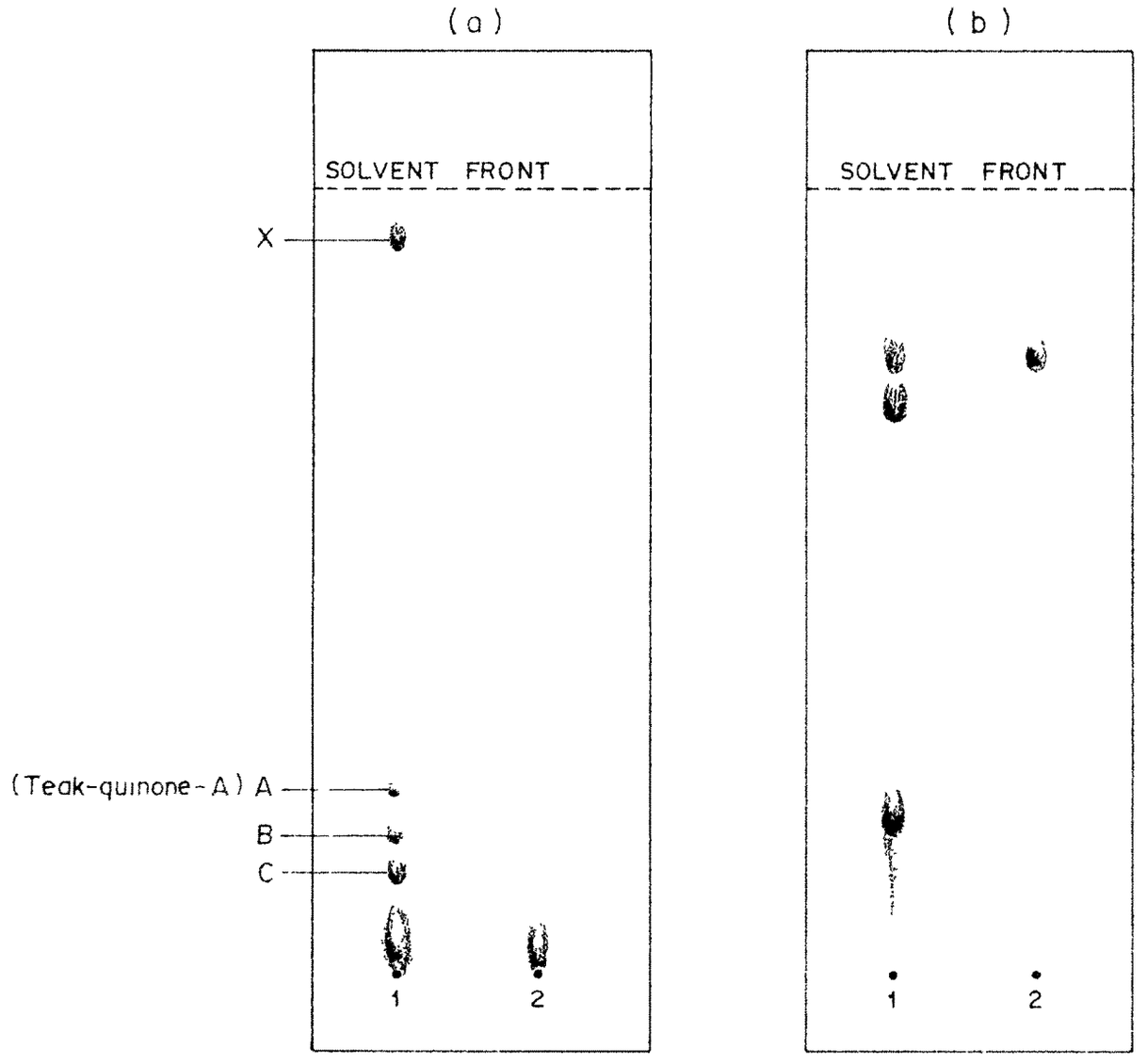
The callus from the third subculture also showed the same type of chromatographic behaviour. The extract from the callus growing in its third subculture was spotted for preparative layer chromatography (PLC) and developed with benzene containing 5% acetone. The PLC showed the presence of six coloured compounds - four yellow, two violet (Fig.8). The major band A was eluted with acetone and filtered, the acetone evaporated to dryness, the residue dissolved in benzene, filtered, concentrated and kept for crystallisation.

The UV spectrum of the crystals was taken in ethanol and their solubility in $NaHCO_3$ and Na_2CO_3 determined. U.V. spectrum was also taken in alkaline sodium dithionite. The pigment turned pink in NaOH solution and on addition of Na-dithionite it turned yellow, which by air oxidation again turned pink.

II. Isolation of teak quinone-A and teak quinone-B

In order to obtain compound A in sufficient amount to identify its structure, a batch of teak callus consisting of about 100 tubes was grown on medium 2 (Table 4).

The tissue was harvested after about 50-60 days of growth. The wet tissue (250 g) was dried in a freese dryer (25 g) and soxhleted successively with petroleum ether, benzene and acetone. Pet.ether removes fast moving and waxy compounds. Teak quinone-A and teak quinone-B were present in benzene extract. Two methods were used for isolation.



1) Teak callus extract

2) 1, 2, 3 trihydroxy 3-Me, anthraquinone

FIG. 8 .

1. Isolation of teak quinone-A by selective solubility

(a) The benzene extracted material (0.3 g) was dissolved in about 50 to 100 cc. of benzene and shaken with a saturated solution of $NaHCO_3$. The $NaHCO_3$ layer was collected and the benzene solution was again extracted. The collected $NaHCO_3$ solution was acidified till slightly acidic, extracted with $CHCl_3$, dried over Na_2SO_4, filtered and concentrated. The emulsion formed did not break easily and was kept for several days.

This $NaHCO_3$ soluble fraction was chromatographed. It showed the presence of traces of teak quinone-A and teak quinone-B on the chromatogram along with four other minor compounds. The presence of teak quinones A and B might be due to traces of Na_2CO_3 in $NaHCO_3$.

(b) Benzene extracted material treated with $NaHCO_3$ was further treated with 5% Na_2CO_3 solution till a colourless extract was obtained. The carbonate solution was acidified, extracted with $CHCl_3$, and washed free of acid, dried over Na_2SO_4, filtered and concentrated.

The presence of teak quinone-A and teak quinone-B was observed in this fraction by chromatography.

(c) The benzene extract after treatment with $NaHCO_3$ and Na_2CO_3 (a & b) was further extracted with 5% NaOH solution till it was colourless. This was then acidified, and extracted with chloroform, the chloroform extract dried over Na_2SO_4, filtered and concentrated. The unbroken emulsion was kept aside.

This fraction on TLC shows the presence of teak quinone-B and other slow moving fractions.

(d) The benzene soluble material shows three bands on preparative layer chromatography.

By preparative layer chromatography, teak quinone-A was collected from $NaHCO_3$ soluble and Na_2CO_3 soluble material. Teak quinone-B was collected from $NaHCO_3$ soluble, Na_2CO_3 soluble and NaOH soluble fractions.

Four mg teak quinone-A (16 mg/100 g dry tissue) was crystallised out from benzene.

2. Isolation of teak quinone-A by chromatography

As described in (II), teak callus was collected and dried (15 g). The powdered tissue was soxhleted successively with pet.ether and acetone. The acetone extract was concentrated and adsorbed on a silica gel, which was loaded on a column (100 cm x 2.5 cm) packed with 90-100 g of column grade silica gel.

The column was eluted successively with benzene, benzene with different percentages of acetone and lastly with acetone. 25 cc. of eluate were collected, teak quinone-A was eluted by benzene containing 5% acetone. This was further purified by preparative layer chromatography and crystallised from benzene.

Five mg. teak quinone-A (30 mg/100 g dry tissue) was crystallised out by this method. Further isolation of the quinone was obtained by this method.

III. Methylation of teak quinone-A

Teak quinone-A (0.050 g) was dissolved in dry acetone and to this 1 g K_2CO_3 and 0.2 ml DMS were added. The reaction mixture was refluxed for 12-14 hrs. After removing acetone by evaporation, distilled water (100 cc) was added to the reaction flask. The separated compound was extracted with chloroform, dried over sodium sulphate, filtered and concentrated to dryness.

This was crystallised from methanol as pale yellow needles (0.04 g).

IV. Demethylation of the methyl ether of teak quinone-A

The methyl ether of teak quinone-A (0.03 g) was dissolved in 1 ml of methyl chloride and treated with 1 ml of 37% HBr in HAc at room temperature for 24 hrs. This was diluted with water and the compound which separated was collected by filtration. The compound was crystallised from benzene (0.02 g) as brown needles.

V. Demethylation of teak quinone-A

Teak quinone-A was treated (0.005 g) with 0.025 ml of 37% HBr in HAc in methyl chloride. The compound was crystallised from methanol in dark brown needles.

VI. Trimethyl silyl ether of teak quinone-A

Teak quinone-A (40 mg) was treated with 1.6 ml of Bis(trimethyl silyl) acetamide at room temperature for 2-3 hrs. The excess of the reagent was removed under reduced pressure. The dry compound was used for NMR studies.

VII. Isolation of compound B_1

After passing through silica gel column and isolating teak quinone-A, the other fractions of the column were monitored on thin layer chromatography.

The fractions showing teak quinone-A and compound B together (1) and compound B and compound C together (2) were further monitored on silica gel by preparative layer chromatography using hexane containing 20% ethyl acetate. Teak quinone-A, compound B and a slow moving compound F were isolated from (1) and compound B, compound C and another compound slower than compound C were isolated from (2).

Compound B (6 mg) was further purified by running a PLC with benzene-ethyl acetate (95:5) where it separated into three bands of very close Rf. The second band B_2 being similar to compound C was mixed with it and kept aside as the total weight of compound was very little. The first band B_1 being major was further purified by running it repeatedly into 100% benzene, where a slow moving minor band was separated.

The compound B_1 (2 mg), being very little and not easily soluble in $CHCl_3$ was subjected to mass spectrum and colour reaction and its structure determined.

SECTION 2

Extraction of Teak Wood

(1) Teak wood powder (2.5 kg) obtained from the local market was extracted with 2 to 2.5 l. of acetone. The acetone extract was concentrated and a TLC run along with teak quinone-A from the callus. The wood extract showed the presence of a compound similar to teak quinone-A.

The acetone extract was adsorbed on spent wood powder and soxhleted successively with hexane and benzene. The benzene extract showed the presence of a compound having similar colour and TLC behaviour to teak quinone-A. This was called TWE-A.

The benzene extract was dissolved in 100-200 ml. benzene, extracted with 5% $NaHCO_3$ and then with 5% NaOH solution till a colourless extract was obtained. Both $NaHCO_3$ and NaOH extracts were acidified and extracted with ethyl acetate. The ethyl acetate extract of $NaHCO_3$ soluble (a) and of NaOH soluble (b) fractions were dried over Na_2SO_4, filtered and concentrated; (b) showed the presence of TWE-A, which was isolated by silica gel column chromatography using hexane, benzene, benzene containing acetone, and acetone.

TWE-A was eluted with benzene containing 2.5% acetone. This was purified by PLC using benzene containing 5% acetone as solvent system. TWE-A was crystallised from methanol as dark coloured needles.

(2) Preparation of ethyl chloroformate of TWE-A

To TWE-A (15 mg) in cold chloroform, ethyl chloroformate (0.2 ml) and pyridine (1 drop) were added. The reaction mixture was kept at 10-15° for 12-16 hrs. The reaction mixture was poured on ice and the solid which separated was extracted with chloroform, dried over Na_2SO_4, filtered, concentrated and separated on PLC into two major bands, one greenish black and one yellow, using benzene as solvent system. The yellow band was collected, eluted with acetone, filtered and concentrated to dryness. The 10 mg of ethyl chloroformate of TWE-A were hydrolysed with 2N NaOH and a yellow precipitate after acidification was obtained which was collected by filtration, crystallised from benzene and sent for mass spectra and IR.

SECTION 3

Synthesis of 1,2,5-trimethoxy-3-methylanthraquinone

Route 1. Preparation of benzoyl benzoic acid. (Chart 1)

(1) 3-Chlorophthalic anhydride (6 g) and cresol methyl ether (4 g) were treated with anhydrous aluminum chloride (10 g) in TCE (100 ml) at 100° and the reaction mass stirred at 110-120° for 6 hrs. The reaction mixture on cooling was poured on 2N HCl in ice. The solvent TCE was removed by steam distillation and the solid which separated on cooling was collected by filtration, washed and crystallised from ethanol (7 g).

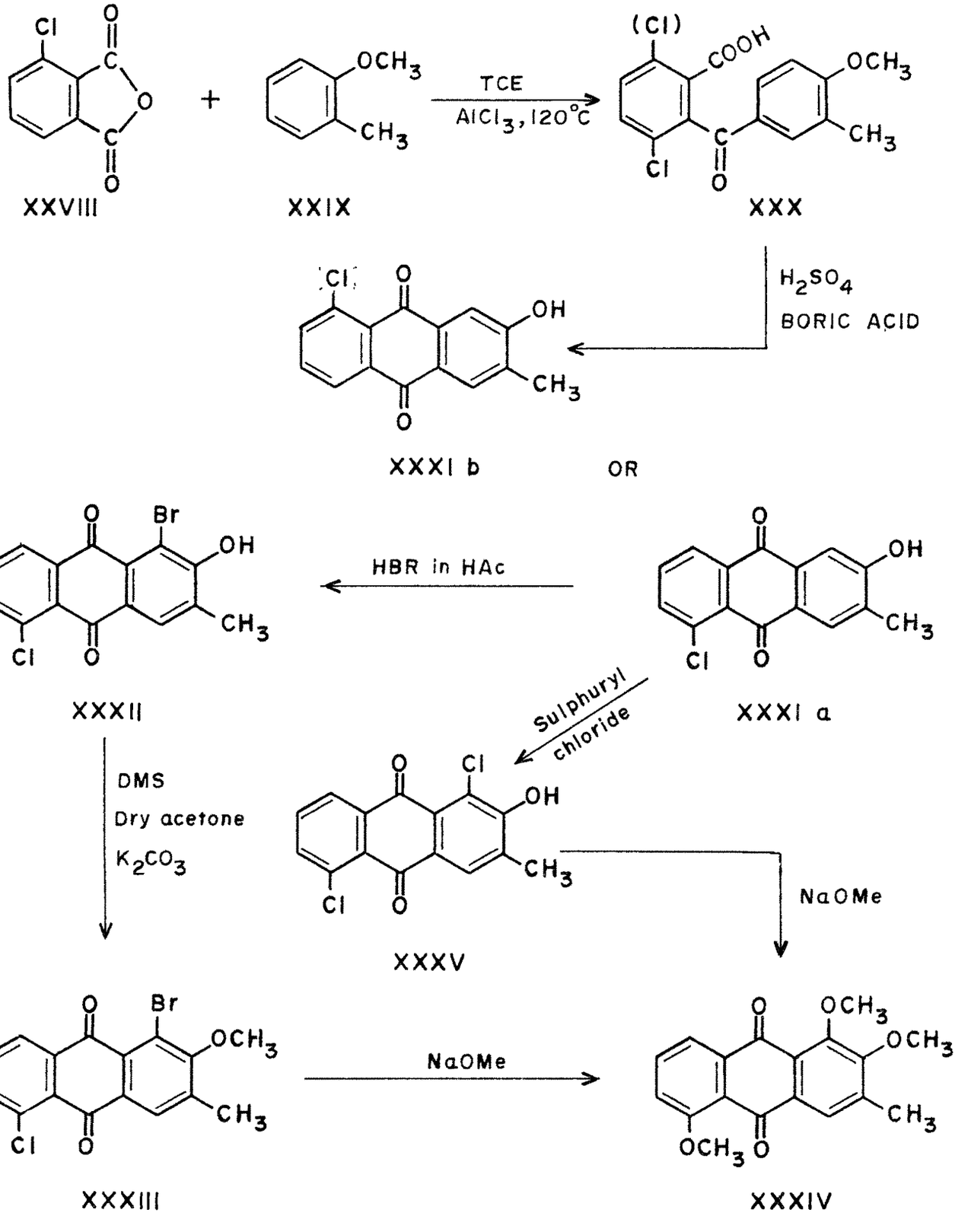
(2) Cyclization of benzoyl benzoic acid

Cyclisation of benzoyl benzoic acid was carried out by (a) NaCl + $AlCl_3$ and (b) H_2SO_4 + Boric acid mixture.

(a) To (10 g) $AlCl_3$ + (2 g) NaCl melt, at 120-130° (1 g) of benzoyl benzoic acid was added. The temperature of the reaction mixture was raised to 180° and kept for 10 min. with stirring. The reaction mixture was allowed to cool to 120° and then poured over ice cold 2N HCl (100 cc). This was kept on a water bath for digestion and the separated solid was filtered, washed with sodium bicarbonate and water and dried. The separated product was dirty green in colour (250 mg).

(b) Benzoyl benzoic acid (1.5 g) was treated with 30 ml of H_2SO_4 and 1.5 g. of boric acid for 2 hours at 140°. The reaction mixture was poured over ice, and the separated solid was

CHART - I



SYNTHESIS OF METHYL ETHER OF TEAK QUINONE-A (Route 1)

collected by filtration. The product was washed with bicarbonate solution to remove keto acid and sulphuric acid and then with water, dried and weighed (0.775 g).

The product (XXXI) was crystallised from acetic acid after treatment with norit. The pale yellow coloured crystals (0.3 g) showed a single spot on TLC, and were soluble in sodium carbonate and had a m.p. 220°.

(3) Bromination of 2-hydroxy-3-methyl-5-chloro-anthraquinone

Compound (XXXI) (0.3 g) was stirred in a 50 ml RB flask containing 15 ml of acetic acid and 0.5 g of anhydrous sodium acetate at 100°. To this a clear bromine solution (1.1 mol) (1 ml bromine diluted with 9 ml acetic acid) was added dropwise and stirred for 3 hrs. The product which separated (XXXII) on cooling was filtered, washed, dried and crystallised from acetic acid (200 mg). The pale yellow crystals had a m.p. 223°.

(4) Methylation of Sample XXX II, i.e. 1-bromo, 5/8 chloro, 2-hydroxy-3-methylanthraquinone

Compound (XXXII) (0.8 g) was refluxed with 1 ml of DMS and 5 g of K_2CO_3 in dry acetone. After refluxing for 12-16 hrs acetone was evaporated off and water was added to the reaction flask. The separated product was collected by filtration, washed with water and dried. The compound (XXXIII) crystallised from methanol (0.5 g) had a m.p. 190-191°.

(5) Methoxylation of 1-bromo, 5/8 chloro, 2-hydroxy 3-methylanthraquinone

Sodium (1.5 g) was dissolved in 100-125 ml of dry methanol. To this freshly prepared methoxide, 600 mg of (XXXIII) and 0.5 g of anhydrous silver nitrate were added and refluxed for 55 hrs. As the reaction progressed some solid separated out. There was no formation of (XXXIV) and the solid was collected by filtration.

In a second set silver nitrate was not added and the reaction carried out as in the first set. Here also the insoluble material separated out, which was collected by filtration. The filtrate on TLC plate showed the presence of two compounds, one corresponding to the solid which was separated and the other corresponding to the methyl ether of teak quinone-A.

The filtrate was diluted with water after removing methanol and extracted with chloroform. The chloroform extract was dried over Na_2SO_4, filtered, concentrated and spotted on PLC. The p PLC was developed with benzene containing 5% ethyl acetate. The four bands which were separated on PLC were collected and eluted with acetone. The compound similar in Rf to the methyl ether of teak quinone-A was about 1-2 mg and was used for mass spectral data.

The solid which separated out was weighed (0.4 g) and analysed. This did not give a halogen test and had a m.p.231-34°C.

(b) Methoxylation by potassium methoxide also gave a poor yield of the desired product.

(6) Preparation of 1,5-dimethoxyanthraquinone

1,5-Dichloroanthraquinone (1 g) was refluxed with 10 ml of phenol and 1 g of anhydrous K_2CO_3 for 6 hrs. This was diluted with ethanol and poured over 10% NaOH solution. 1,5-Diphenoxy anthraquinone (1 g) having a m.p. 213-215°C was dissolved in 100 ml dry methanol and refluxed with 5 g of KOH and 5 ml of pyridine for 60 hrs. The separated product was crystallised from methanol as pale yellow needles having a m.p. 236°C, which was identical to 1,5-dimethoxyanthraquinone.

(7) Phenoxylation of 1-bromo,5-chloro-2-methoxy-3-methyl-anthraquinone

Compound (XXXIII) (0.8 g) was taken with 0.8 g K_2CO_3 and 8 ml of phenol and refluxed for 6 hrs in a 100 ml RB flask. This was diluted with ethanol and poured over 2N NaOH solution, till it was alkaline. The product did not separate out. The solution was extracted with chloroform and the extract spotted on TLC. This showed many spots and a black material which did not move on TLC.

(8) Preparation of 1,2,3,4-tetramethoxyanthraquinone

1,3-Dihydroxyanthraquinone was brominated with Br in acetic acid containing fused Na-acetate. The product obtained 1,3-dihydroxy 2,4-dibromoanthraquinone(m.p. 224-226°C) was methylated with DMS and then subjected to phenoxylation as described in (7).

The reaction product when worked out showed 4 spots on TLC with trailing, and had black appearance.

(9) Preparation of 1,5-dichloro,2-hydroxy-3-methylanthraquinone

Compound (XXXI) (100 mg) was dissolved in 30 ml of dry benzene. To this 0.03 ml of sulphuryl chloride was added and refluxed for 4 hrs. On TLC plates it showed two spots with benzene as solvent system. To the reaction mixture 0.015 ml of sulphuryl chloride was again added, refluxed for 4 hrs. and then benzene was distilled off. The product was crystallised from methanol and mass spectra taken.

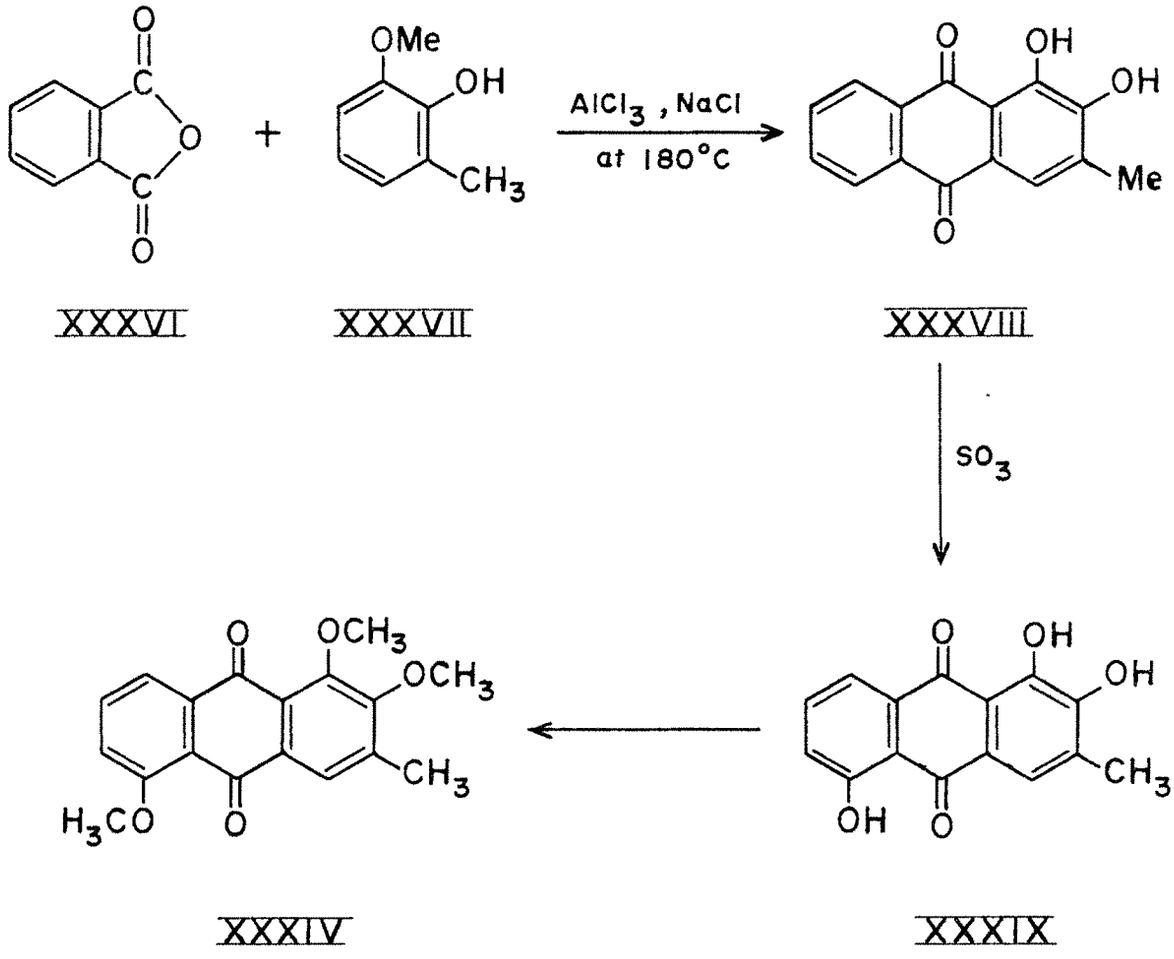
The molecular weight of the product was 272 which is similar to (XXXI).

Route 2:

(1) Preparation of 3-methyl alizarin (chart - 2)

At 180°C a clear melt of anhydrous $AlCl_3$ (100 g) and NaCl (20 g) was made and the temperature was bought down to 120-130°C. At this temperature, a homogeneous melt of phthalic anhydride (XXXVI) (9 g) and 3-methyl catechol monomethyl ether (XXXVII) (7 g) was added in lots with stirring. The temperature was raised to 180°C and maintained for half an hour. The reaction mixture was allowed to cool down to 120° and then poured into 2N HCl in ice (1.5 l.). This was digested on a steam bath for half an hour, filtered, washed and dried. The crude product on oxalated silica gel plates using benzene as the solvent system showed a yellow spot. The compound was extracted with hot benzene 3 to 4 times. The

CHART-2



XXXVI XXXVII XXXVIII

XXXIV XXXIX

SYNTHESIS OF METHYL ETHER OF TEAK QUINONE-A,-ROUTE-2

hot benzene solution was treated with norit, filtered and concentrated to 50 ml. The (3 g) of benzene soluble material gave 1 g pure crystalline product which had a m.p. 245°C (3-methyl alizarin, m.p. 250/252°C (XXXVIII).

II. Preparation of 3-methyl, 1,2,5-trihydroxyanthraquinone

3-Methyl alizarin (1 g) was treated with 6 ml of 100% oleum at room temperature in a 50 ml stoppered RB flask for 14-18 hrs. The reaction mixture was diluted with 50 ml of concentrated H_2SO_4 and poured on 250 g crushed ice. The sulfuric ester which separated was filtered and the wet cake dissolved in 10% aqueous NaOH solution. The violet solution was acidified carefully with concentrated H_2SO_4, avoiding an excess of the acid. On boiling for 15 minutes, the precipitate was filtered and washed well. The dry precipitate (0.750 g) showed two spots, on oxalated silica gel plates using benzene as solvent. One corresponded to 3-methyl alizarin. The crude compound (0.750 g) was dissolved in DMF and absorbed on column grade silica gel. A dark violet band was eluted from the column upon elution with benzene. The eluate was concentrated to 1-2 ml and 5 ml of methanol were added to it. The compound crystallised out when allowed to stand overnight. The crystalline product (180 mg) was 1,2,5-trihydroxy-3-methyl-anthraquinone (XXXIX), while the mother liquor contained mostly 3-methyl alizarin. The crystals were bright red microplates having a m.p. 220°C.

	Theory	Found
C	66.7%	67.2%
H	3.7%	4.5%.

III. Methylation of 1,2,5-trihydroxy,3-methylanthraquinone

1,2,5-Trihydroxy-3-methylanthraquinone (0.3 g) was refluxed with DMS, acetone and dry K_2CO_3 for 6-8 hrs. After working up the reaction mixture, the crude product was dissolved in $CHCl_3$ and passed through a grade I neutral alumina column. The eluate was concentrated and spotted on PLC, which was developed with a mixture of benzene-acetone (9:1) and out of the three bands the major band was collected, extracted with acetone and afterwards evaporated to dryness. The compound was crystallised from methanol as yellow needles (180 mg) and had a m.p. 130-137°C.

	Theory	Found
C	69.2%	69.0%
H	5.1%	5.3%

The IR spectrum of the synthetic compound is superimposable with that of the dimethyl ether of the compound isolated from teak callus culture.

The fast moving band from the PLC gave yellow needles melting at 131-132°C which was near the melting point of 3-methyl alizarin dimethyl ether (m.p. 127°C).

SECTION 4

DISCUSSION

1. Structure of teak quinone-A

In the present work, the isolation of the pigments from teak callus tissues and characterisations of these compounds has been discussed.

The tissues were lyophilised and extracted with acetone. The acetone extract on thin layer chromatographic plates (silica gel; benzene-acetone) showed the presence of four pigments designated as teak quinones A, B, C and D. D moves very fast on TLC plates (Fig. 8).

Initially functional separation of the total acetone extract was tried but was unsuccessful. Thus when the total extract was shaken successively with aqueous saturated $NaHCO_3$, 10% aqueous Na_2CO_3 and 5% aqueous NaOH, there was no clear cut separation of these compounds. All the pigments were soluble in aqueous Na_2CO_3.

The total extract was submitted to silica gel column chromatography using benzene and benzene-acetone for development and elution of the fractions. Acetone was used in increasing percentage in benzene and a number of fractions were collected. All the fractions were monitored on TLC plates and similar fractions were pooled and worked up in the usual manner. The first few fractions contained the major compound (teak quinone-A). It was crystallised from benzene as yellow needles, m.p. 237°C.

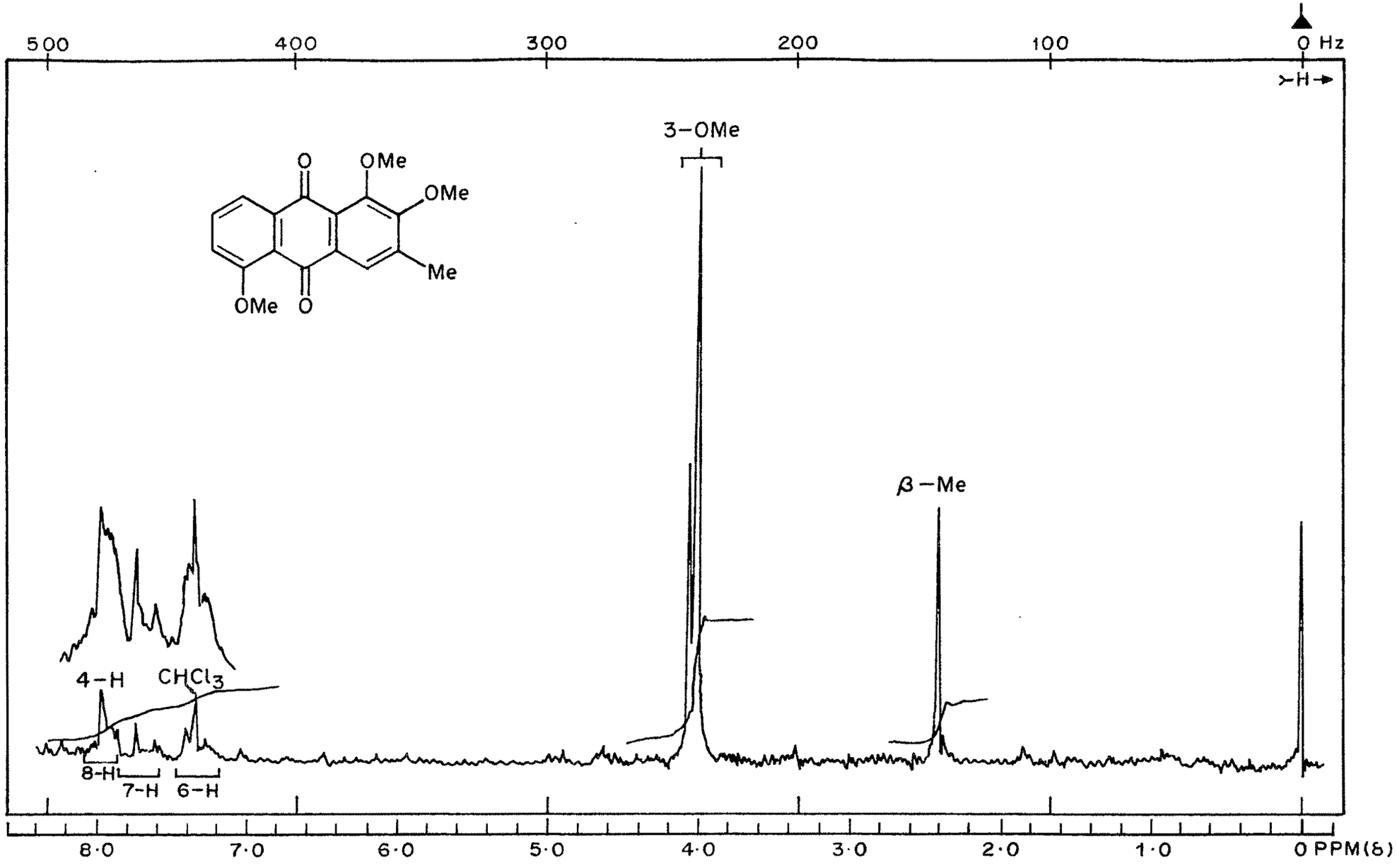


FIG. 9 NMR OF METHYL ETHER OF TEAK QUINONE-A

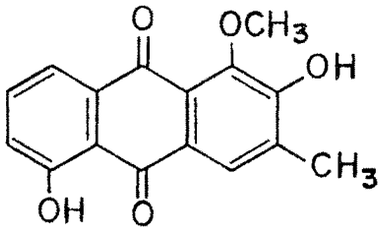
It was insoluble in aqueous sodium bicarbonate, but dissolved in 5% aqueous sodium carbonate. It was readily reduced by alkaline sodium dithionite and was regenerated by air oxidation, indicating its quinonoid character. The electronic spectrum of the pigment in alkaline sodium dithionite showed that it is an anthraquinone derivative, and its solubility in aqueous sodium carbonate indicated that it has a hydroxyl group in β -position. The mass spectrum showed the molecular ion peak at m/e 284, shifted by in situ deuteration to m/e 286, showing the presence of two hydroxyl groups. The IR spectrum (KBr pellet) showed the presence of a hydroxyl group (3350 cm^{-1}), a non-bonded carbonyl (1675 cm^{-1}) and a bonded carbonyl (1635 cm^{-1}). The UV spectrum in ethanol showed maxima at 253, 276 and 415 nm.

Its NMR spectrum (pyridine, chemical shifts on τ -scale) showed the presence of one methoxyl group (6.1) and a methyl group (7.6) in a β -position of an anthraquinone molecule. On methylation with methyl sulphate and potassium carbonate in boiling acetone, it gave a dimethyl ether (M^+ 312) indicating the presence of two hydroxyl groups. The NMR spectrum in $CDCl_3$ (Fig. 9) of the dimethyl ether shows the presence of a β-methyl group at 7.6 and three methoxyl groups at 5.95-6.05. In the aromatic region it shows a single-proton quartet at 2.75, a single-proton triplet at 2.33 and a single-proton quartet at 2.15; the pattern is characteristic of the aromatic protons of 1,8-dimethoxyanthraquinone. In addition to these three aromatic protons, the spectrum shows a single-proton singlet at 2.1

(overlapping with the quartet at 2.15) indicating that one of the benzene rings of the anthraquinone molecule is substituted in the 1,2,3-positions. The occurrence of the singlet at 2.1 about 0.4 ppm downfield compared with the 4-proton in 1-methoxy-3-methylanthraquinone can be explained by the overcrowding of the adjacent groups which may result in pushing α-methoxyl group out of the molecular plane.

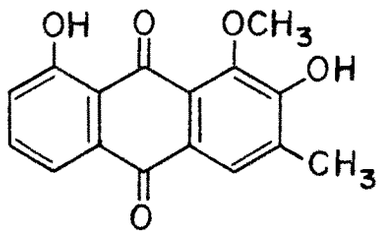
The trimethylsilyl ether of teak quinone A has been prepared by dissolving it in excess of bis-(trimethylsilyl)-acetamide and then distilling the excess of the reagent. The NMR spectrum of the silyl derivative in CCl_4 showed two, three proton singlets at 7.7 and 6.2 representing the β-Me and OMe groups respectively. In the aromatic region it shows the presence of 4 aromatic protons, a quartet at 2.93, a triplet at 2.47 and another quartet at2.17 all constituting ABC spectrum indicating that one of the benzene rings of the anthraquinone moiety is substituted at 1-position by an OH or OMe group. Besides these three protons a singlet at 2.13 suggests that the other benzene ring is substituted at 1,2,3-positions.

The substitution in one benzene ring in the dimethyl ether can therefore be 1,2-dimethoxy-3-methyl or 1,3-dimethoxy-2-methyl, the former is to be preferred because the methyl sandwiched between two OMe groups will appear at 0.2 to 0.3 ppm upfield compared to a normal β-methyl group. Further proof of the former orientation of groups in one ring was obtained by



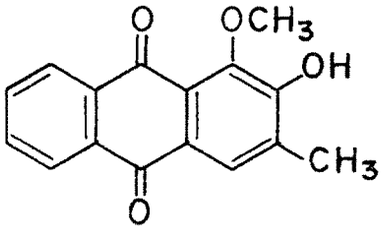
XL

TEAK QUINONE-A



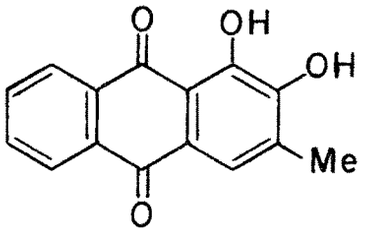
XLI

OBTUSIFOLIN



XLII

DIGILOLUTEIN



XLIII

3-METHYL ALIZARIN

treatment of the parent pigment with hydrobromic acid in glacial acetic acid; the product was a trihydroxy-methylanthraquinone which gave a colour reaction characteristic of alizarin (violet with aqueous sodium hydroxide). Based on the spectral and chemical evidence, teak tissue quinone-A can have structure (XL) or (XLI).

Structure (XLI) was suggested earlier for obtusifolin, a compound isolated from Cassia obtusifolia (Takido 1960) and its dimethyl ether was synthesised by an unambiguous route by Patwardhan (1961). The dimethyl ether of the teak tissue quinone was not identical with the dimethyl ether of (XLI) (TLC behaviour, m.p., IR). The dimethyl ether of the new quinone, on treatment with hydrobromic acid in acetic acid at room temperature, which preferentially demethylates α-methoxyl groups in anthraquinone, gave a dihydroxy compound whose IR spectrum shows only one absorption at 1635 cm^{-1} for both the carbonyls indicating that the hydroxyls are in the 1,5-position. Further, its NMR spectrum ($CDCl_3$) shows the signals corresponding to the bonded OH groups at -2.83 and -2.63, which can be assigned to the hydroxyls in the 1- and 5-positions respectively. Hydroxyls in the 1,8-positions may be expected to appear in the region around -2.0.

The mass spectrum of teak quinone-A (Fig.10) showed a strong peak at m/e 284 (base peak) corresponding to the molecular ion. There were relatively few peaks in the spectrum. The molecular ion loses 18 mass units giving an intense peak at

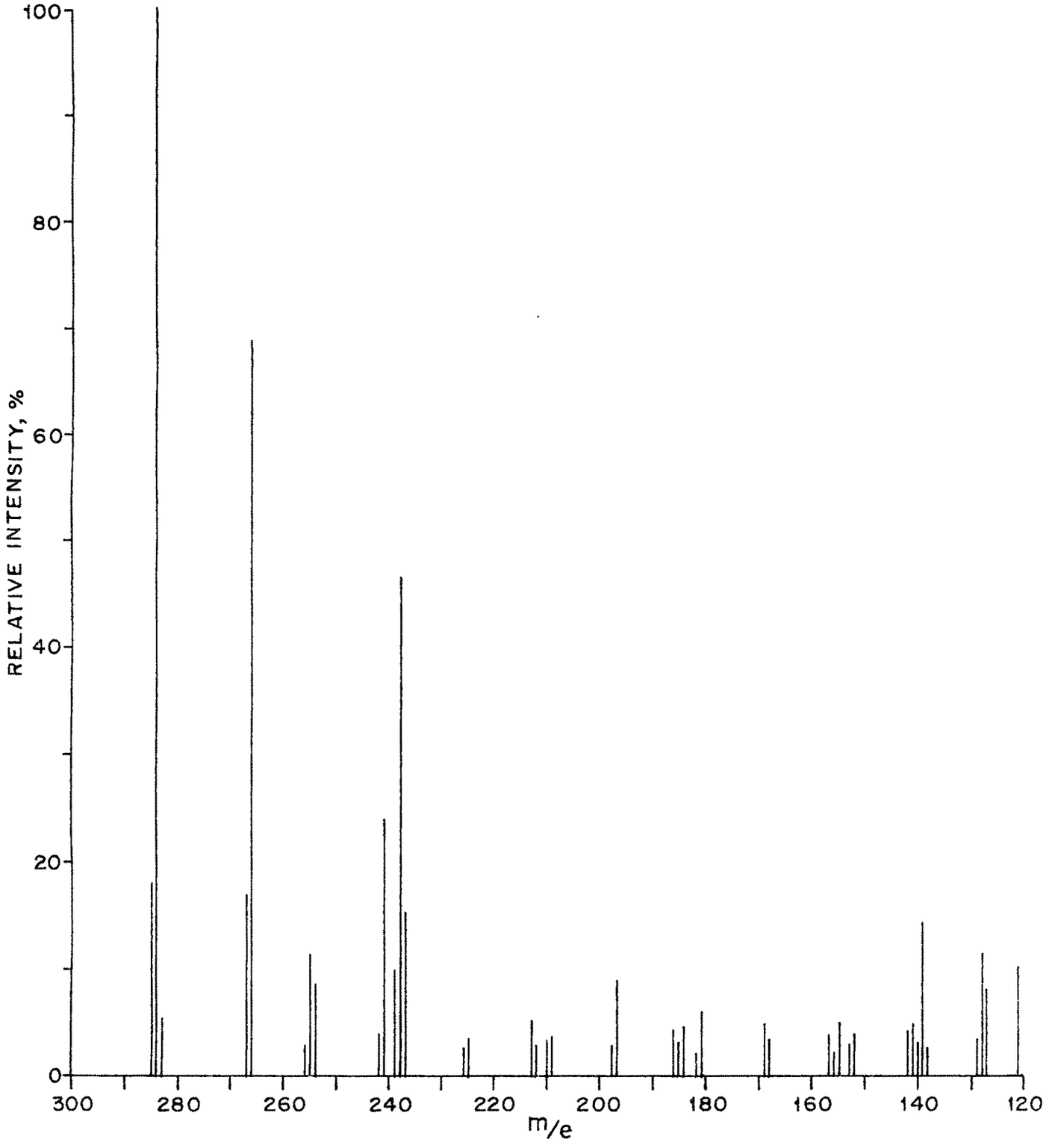


FIG. 10 TEAK QUINONE – A

CHART-3

MASS SPECTRAL FRAGMENTATION OF TEAK QUINONE-A

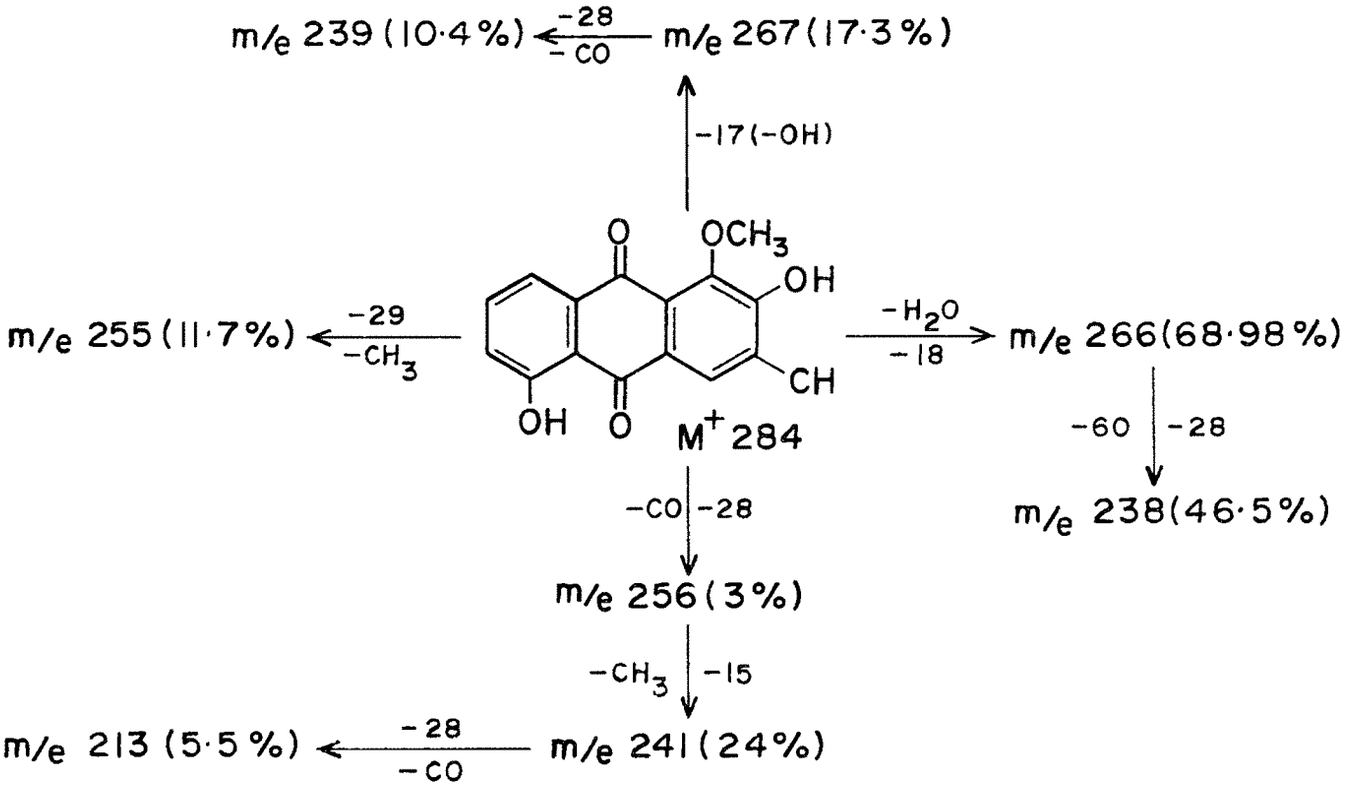
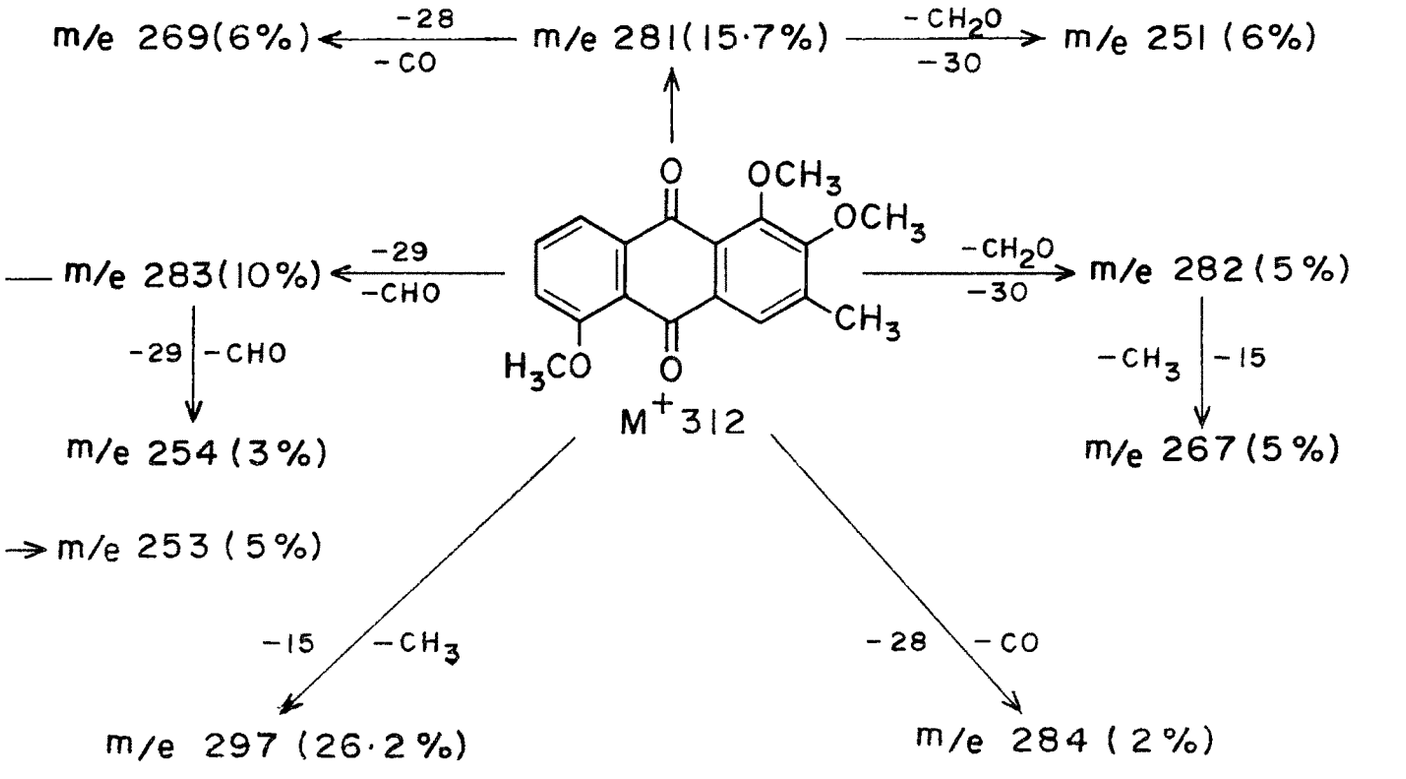


CHART-4

MASS SPECTRAL FRAGMENTATION OF DIMETHYL ETHER OF TEAK QUINONE-A



m/e 266. It shows as the other hydroxy and methoxy anthraquinone compounds loss of carbonyl, methyl and hydroxyl groups (Chart 31). In the mass spectrum of dimethyl ether of teak quinone A (Chart 34) also the base peak corresponds to a molecular ion. The peak corresponding to M-15 at m/e 297 is relatively of low intensity compared with the 1,8-dimethoxyanthraquinone supporting that the methyl groups must be in 1,5-positions.

Teak quinone-A, therefore, has the structure (XL). When this work was completed there was no record of the occurrence of an anthraquinone derivative in a tissue culture; but in recent papers isolation of 3-methylpurpurin-1-methyl ether, an isomer of (XL) from the tissue culture of Digitalis lanata has been reported (Furuya et al. 1971, 1972). Alizarin, morindone and damacanthal have been isolated by Leistner from the callus of Morinda citrifolia (Leistner 1973). None of the quinones so far isolated from teak has the structure (XL).

NMR spectra of hydroxyanthraquinones

A number of hydroxyanthraquinones have been isolated from various natural sources and in recent years their structures have been elucidated mainly based on their spectral data. NMR analysis of these compounds is very helpful in arriving at the structures of these compounds although it may not be possible to arrive at the location of different substituents in the two benzene rings of the anthraquinone molecule with certainty. Thus it is difficult from the NMR data to differentiate the structure of 1,6-dihydroxyanthraquinone from 1,7-dihydroxyanthraquinone.

Anthraquinone shows two sets of multiplets centered at 1.8 and 2.2, corresponding to the α and β protons respectively. In general an OH group shifts the ortho and para protons by 0.5-0.6 ppm upfield while a Me group shifts by 0.3 ppm. The rule of additivity is very much valid in these compounds. Methoxyl group shifts the ortho and para positions similar to hydroxyl substituents. Thus in 1-hydroxy anthraquinone the 2-protons appear around 2.8, an upfield of 0.6 ppm. In 1,3-dihydroxyanthraquinone the 2-H is seen at 3.3, while the 4-H is at 2.8, both have moved to higher field by 1 ppm compared to the values in unsubstituted anthraquinone. However, the rule of additivity is not valid in the case of 1,2,3-trimethoxy-anthraquinone and 1,2-dimethoxy-3-methylanthraquinone, because the 4-H is always seen at a lower field than expected. Thus in teak quinone-A, the 4-H is seen at 2.1 and not at 2.5 to 2.6 which is normally expected if the rule of additivity is valid. This can be explained by overcrowding of the substituents resulting in pushing the 1-OMe group out of the molecular plane. However, if this is partially demethylated, the α-OH group will be bonded with the adjacent C=O group and will come in plane with the rest of the molecule and the 4-H appears as usually expected.

No proper attempt has been made in analysing the OH groups in hydroxyanthraquinones in their NMR spectra. These compounds are normally insoluble in $CDCl_3$, but they go freely in DMSO. The OH groups at α-protons are seen as sharp

Table - 35

Chemical shift of the bonded hydroxyls in some of the hydroxyl derivatives of anthraquinone

No.	Compound	Solvent	Chemical shift	Multiplicity	No. of protons	Possible assignment
1	1-Hydroxyanthraquinone	DMSO	-2.3	s	1	chelated OH
2	2-hydroxyanthraquinone	"	-1.15	s	1	β-OH
3	1,3-Dihydroxyaq.	"	-2.9	s	1	chelated OH
4.	1,4-Dihydroxyaq.	$AsCl_3$	-2.88	s	2	"
5	1,8-Dihydroxyaq.	$CDCl_3$	-1.84	s	2	"
6	1,2,4-trihydroxy	DMSO	-3.3	s		
7	1-Hydroxy,2-hydroxy methyl,3-methoxyaq.	$CDCl_3$	-2.70	s	1	"
8	1-Hydroxy,3-methyl aq.	"	-2.60	s	1	"
9	1,8-Dihydroxy,3-methyl aq.	DMSO	-2.80	s	1	"
10	1,2-Dihydroxy, 4-methyl aq.	"	-3.3	s	1	"
11	1,8-Dihydroxy, 3-methyl aq.	$CDCl_3$	-1.90 -1.80	s	1	"
12	1,5-Dihydroxy-2-methoxy,3-methyl aq.	DMSO	-2.83	s	2	"

signals because of their bonding with the C=O groups, but the β-OH groups many times appear as broad signals and also difficult to assign if there are more than one such substituents. In 1-hydroxyanthraquinone, the OH groups is seen at -2.9, and the variation is not much with different substituents (see Table 35). However, if there are two hydroxyls, both at α-positions in the same ring or different rings, the situation is altogether different and one can easily find out the location of the hydroxyls. If the OH groups are at 1,4-positions as in quinizarin and purpurin they are seen around -3.2 to -3.5. But if the two hydroxyls are in the different rings of the anthraquinone moiety then two isomeric structures can be written, i.e. 1,5- or 1,8-substituted derivatives and the chemical shifts of their OH groups are a diagnostic feature in differentiating these isomers. Thus in 1,5-dihydroxyanthraquinone, the OH groups are strongly bonded with the C=O groups and appear around -2.7 to -3.3. However, if they are located at 1,8-positions, then both are bonded with the same C=O group and are seen at -1.8 to -2.1. A few such examples have been quoted in Table 35. Teak quinone-A is partially demethylated with HBr-CH_3COOH, the NMR spectrum of the resultant 1,5-dihydroxy-2-methoxy-3-methylanthraquinone shows the bonded OH groups at -2.63 and -2.83. This supports the orientation of the oxygenation pattern in teak quinone-A at 1,5- and not at 1,8- position.

(2) Teak quinone B1

As described in the experiments, compound B_1 was isolated and purified by repeated chromatography. The quantity of compound B_1 being very little (less than 2 mg) it was subjected to mass spectra only. It shows a molecular ion peak at 268. A strong peak is shown at M-15 indicating the presence of one methoxyl group.

The compound gave positive vat test indicating its quinonoid nature. The absence of ferric colour, shows that there are no α hydroxyl groups. Its solubility in Na_2CO_3 indicates the presence of a β-hydroxyl group. From the molecular weight it can be assumed that the present compound is either digitolutein (1-OCH_3, 2-OH, 3-CH_3 anthraquinone)or 3-methylalizarin-2-methyl ether, 1,5 or 1,2-dimethoxyanthraquinone, all being very near to teak quinone-A. The last three possibilities were ruled out as β-OH is indicated by solubility in aq. Na_2CO_3. Also on demethylation with HBr in HAc, the compound gives a dark violet colour with NaOH solution showing the presence of two adjacent OH groups. Its melting point (215-218°) being nearer to digitolutein (224-228°) the compound was found to be digitolutein, which for the first time is being reported in teak(XLII)

(3) Presence of 1,2,5-trihydroxy-3-methylanthraquinone in teak tissue culture

Total acetone extract of teak tissue indicated a violet band near the base line when chromatographed on silica gel plates. A pure sample of 1,2,5-trihydroxy-3-methylanthraquinone was spotted

along with the total teak tissue extract. RF values of both the 1,2,5-trihydroxy-3-methylanthraquinone and the violet band were similar in benzene-acetone (95:5) and benzene-ethyl acetate(95:5) systems. The total mixture and 1,2,5-trihydroxy-3-methyl were spotted on oxalated silica gel plates, and it was noted that the total mixture contained a compound similar to 1,2,5-trihydroxy-3-methyl anthraquinone. From this it can be concluded that the teak tissues contain the latter compound.

4. Chemical examination of teak wood

The presence of teak quinone-A in the teak tissue culture required reinvestigation of teak wood to ascertain its presence or absence of the same. Sandermann and Simatupang (1966) have reported 7 anthraquinones from teak wood. None of the reported compounds is identical to teak quinone-A (Fig.7).

From the acetone extract of teak wood a compound having similar Rf to that of teak quinone-A was obtained. This compound (TWE-A) was isolated and purified by column chromatography and PLC (Benzene-acetone, 95:5). TWE-A was crystallised from methanol as dark yellow crystals. TWE-A gave a positive vat test indicating its quinoid nature. Further purification of this compound was done by preparing an ethylchloroformate derivative.

The NMR spectrum of the above derivative was taken in $CDCl_3$. It indicates a β-methyl at 7.5 and a methoxyl at 5.95 and a singlet at 1.9. Intensityof methyl was double that of methoxy

indicating that this compound is probably a mixture of two compounds. When spotted on an oxalated plate and run with benzene containing 5% acetone, it showed the presence of two compounds. From this data the following two structures can be suggested for these compounds. Teak quinone B_1 (XLII) was also isolated from callus cultures.

(5) Synthesis of 1,2,5-trimethoxy-3-methylanthraquinone

The synthesis of teak quinone-A trimethyl ether has been undertaken to confirm its structure. (chart 1 & 2)

In the first instance an attempt has been made to synthesise this compound by the condensation of 3-chlorophthalic anhydride with o-cresol methyl ether and subsequent bromination and conversion of the two halo groups to methoxyls as indicated in chart 3.

3-Chlorophthalic anhydride was prepared by bubbling chlorine gas through a melt of 3-nitrophthalic anhydride at 240°. Condensation of o-cresol methyl ether with 3-chlorophthalic anhydride resulted in a mixture of two benzoyl benzoic acids, one being major, and were cyclised with sulphuric acid-boric acid to yield the corresponding anthraquinone derivatives (XXXI). However, when the crude mixture was crystallised, the major compound separated and was homogeneous on TLC. The product is soluble in aqueous sodium hydroxide solution indicating that it is a α-hydroxyanthraquinone derivative and may have formed by demethylation during the cyclisation process.

It can be represented either as 2-hydroxy-3-methyl-5-chloro-anthraquinone (XXXIa) or 2-hydroxy-3-methyl-8-chloroanthraquinone (XXXIb). This on bromination gave the corresponding 1-bromo derivative (XXXII). The product was methylated with dimethyl-sulphate and K_2CO_3 in boiling acetone to give 1-bromo-2-methoxy-3-methyl-5 or 8 chloroanthraquinone (XXXIII). The NMR spectrum shows two singlets of 3 protons each at 6.09 and 7.75 corresponding to OMe and a -methyl group of the anthraquinone moiety.

Compound (XXXIII) was then treated with sodium methoxide in absolute methanol in presence of silver nitrate. The reaction product was found to be a mixture of compounds and did not contain any spot corresponding to the Rf value of teak quinone-A or obtusifolin dimethyl ether. On purification by column chromatography, small quantity of a pure product was isolated and was characterised as 2,5-dimethoxy-3-methylanthraquinone formed by the debromination of (XXXII) during the reaction. However, if the conversion of the halo groups to methoxyl was carried out in the absence of silver nitrate, the reaction mixture showed the presence of a minor compound corresponding to the Rf value of teak quinone-A. The compound was too small in quantity that no further attempt could be made to obtain the product by this route.

It is known that chlorine or bromine can be converted with sodium phenoxide to the corresponding phenoxy derivative more easily than directly to OMe groups. Further the phenoxyl

groups can be exchanged with OMe groups by boiling with sodium methoxide in absolute methanol. When this sequence of reactions were carried out on 1,5-dichloroanthraquinone, the resultant 1,5-dimethoxyanthraquinone was obtained in reasonable yields, but in the case of 1-bromo-2-methoxy-3-methyl 5 or 8 chloroanthraquinone, the same sequence of reactions resulted in intractable mixture of products.

Having failed by the usual sequence of reactions indicated above, the synthesis of teak quinone-A methyl ether has been achieved unambiguously starting from 1,2-dihydroxy-3-methyl-anthraquinone. (Chart 2)

About 83 years ago Bohn (1891) treated alizarin with a high content of oleum and obtained a valuable dye which was subsequently characterised as 1,2,5,8-tetrahydroxyanthraquinone. Almost at the same time and independently Schmidt (1896) came across ~~across~~ the same reaction using 2% oleum. It was Gatterman (1891) who examined the dye obtained from alizarin and found it to be 1,2,5,8-tetrahydroxyanthraquinone. Schmidt also found simultaneously a number of other methods of introducing OH group into the anthraquinone moiety. This caused some misunderstanding about a clear definition of the Bohn-Schmidt reaction and is referred to the action of a high percentage of oleum with hydroxyanthraquinone with atleast one hydroxyl at 2-position at temperature between 25-30°. Although this method of hydroxylation is basic and of technological importance in anthraquinone chemistry,

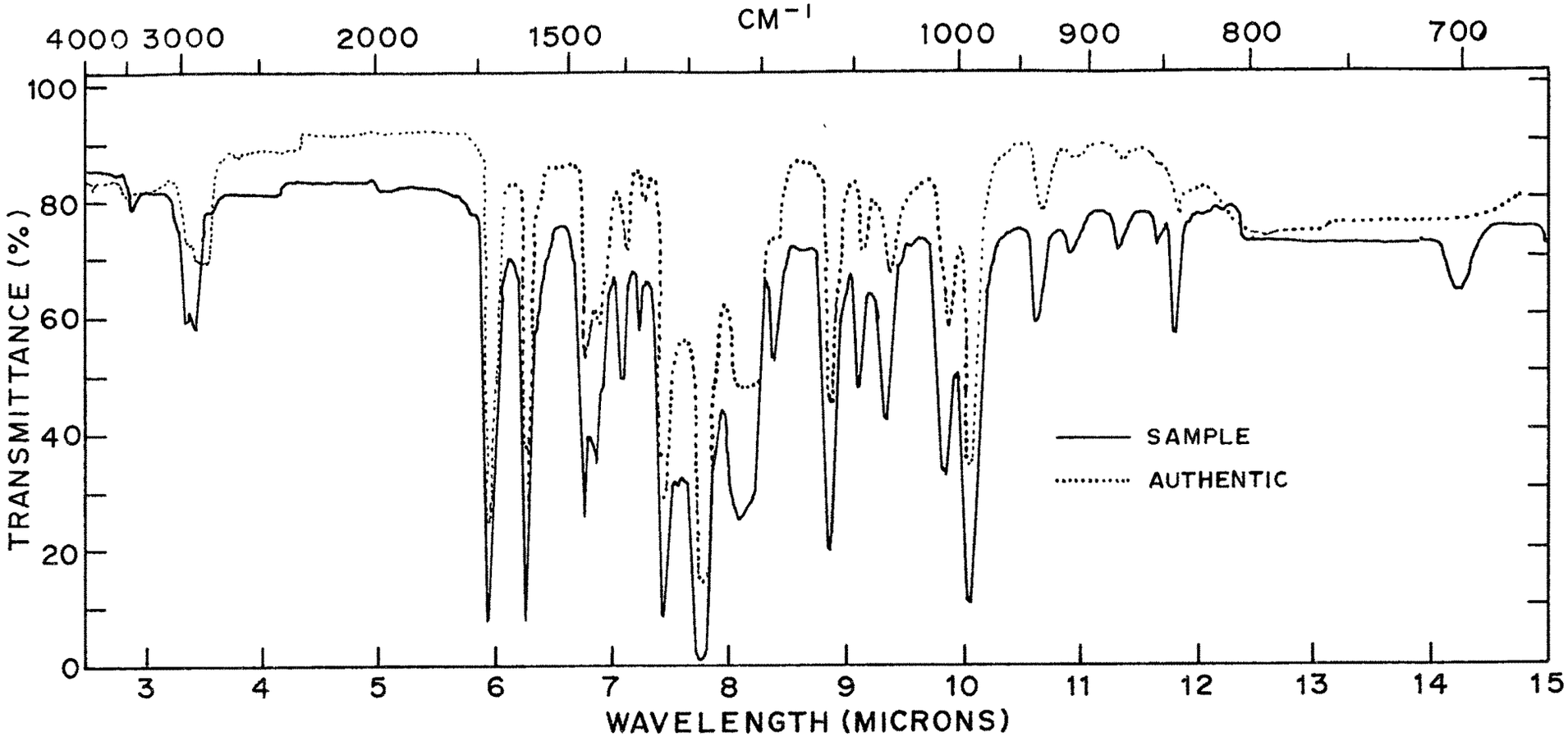


FIG. 11 IR OF METHYL ETHER OF TEAK QUINONE A and SYNTHETIC PRODUCT

there are only scanty references in the literature about the nature of this reaction. According to Gattermann (1891) the conversion of alizarin to quinalizarin goes through a neutral cyclic sulphate with as intermediate. The isolation of this cyclic sulphate gives no indication of the reaction course. The mechanism of the reaction is also difficult to predict at this stage. It is also found that if the reaction is carried out for a shorter duration of time the product from alizarin will be 1,2,5-trihydroxyanthraquinone. From this it is clear that alizarin goes to quinalizarin through 1,2,5-trihydroxy-anthraquinone by further hydroxylation.

It was thought that 1,2,5-hydroxy-3-methylanthraquinone can be prepared by Hohn-Schmidt's reaction on 1,2-dihydroxy-3-methylanthraquinone (XXXVIII). This compound was synthesised by the condensation of 3-methylcatecholdimethyl (XXXVII) ether with phthalic anhydride (XXXVI) by aluminium chloride-sodium chloride melt. The compound (XXXVIII) was then dissolved in 80% oleum and left at 25-30° for 12-16 hr. and on working up, 1,2,5-trihydroxy-3-methylanthraquinone (XXXIX) was obtained together with some starting material. The product showed in the IR spectrum both the C=O groups bonded to the hydroxyl groups and hence the orientation of hydroxyl were fixed. On methylation it gave 1,2,5-trimethoxy-3-methyl (XXXIVa) anthraquinone identical with the dimethyl ether of teak quinone-A. The IR spectra of teak quinone-A trimethyl ether and the synthetic product are superimposable (Fig. 19).

C H A P T E R - V

EFFECT OF VARIOUS FACTORS ON THE PRODUCTION OF TEAK QUINONE-A AND ITS BIOGENESIS

***

SECTION 1

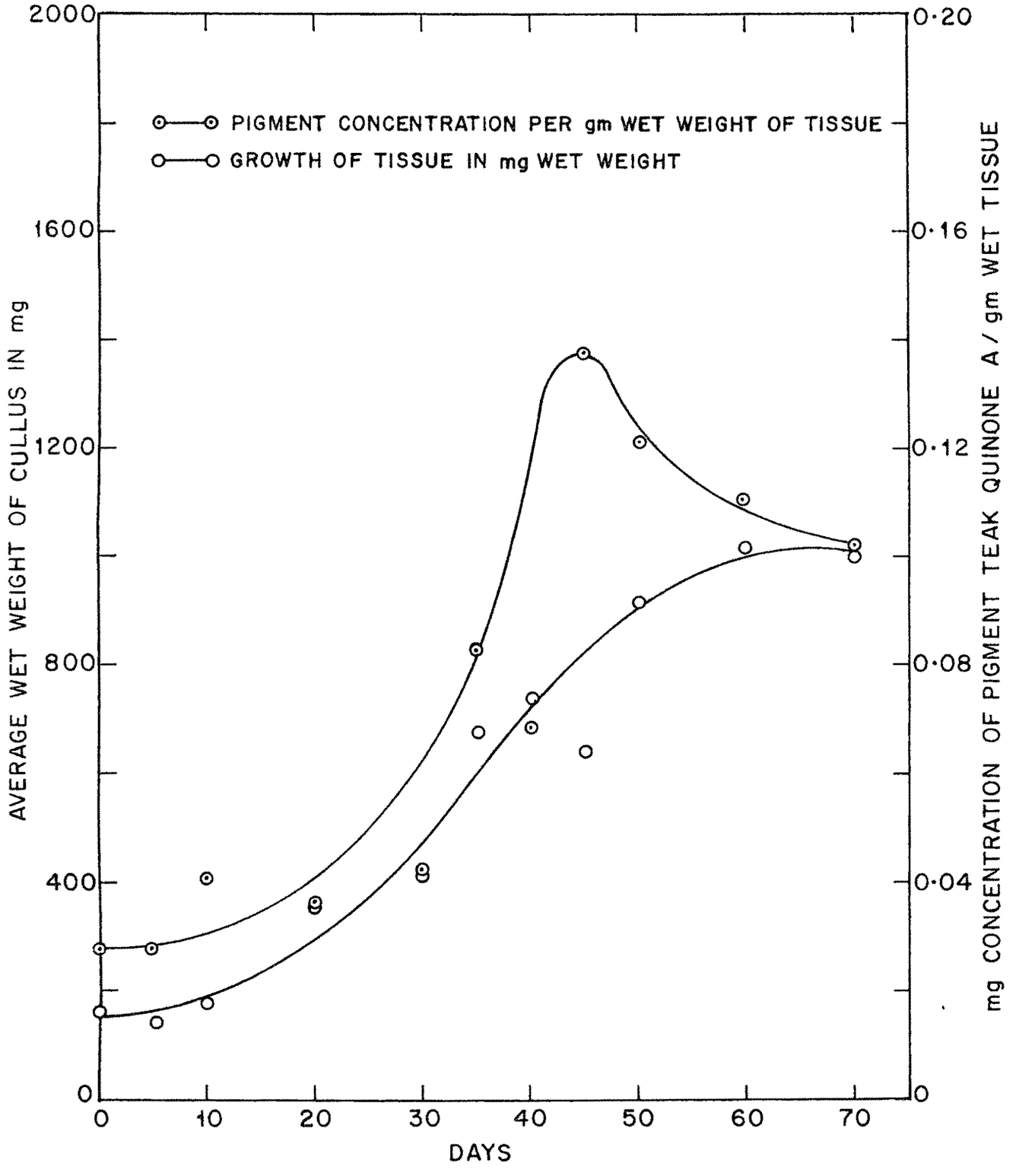
Effect of various factors on the production of Teak quinone - A.

Experimental and Results

In Chapter III, the effect of various growth factors on the growth of teak callus was described. This chapter deals with the effect of some of these factors on pigment production, particularly of teak quinone-A whose structure has been discussed in Chapter IV. The tissue was grown as described in Chapter I on different media containing the growth substances, and harvested after 35-45 days. Estimates were made as described in Chapter IV and the products, chiefly teak quinone A, isolated by PLC using benzene, acetone (95:5). For quantitative determination, comparisons of the u.v. absorption at 276 or 410 nm were made against known amounts of crystalline teak quinone A. The effect of a particular growth factor on the production of pigment was compared with controls in each set of experiments. Changes in the composition of the media with respect to the different test substances are referred to in the respective Tables. Chromatographic pattern is compared with that of Fig.8a (spot 1).

(a) Effect of age of tissue

Callus tissue for these studies was obtained by growing the tissue on the standard medium containing 10 ppm gibberellic acid and 3:2 ppm glycine instead of 2 ppm since the earlier isolation of teak quinone A was carried out from tissue grown on this medium. Pigment (Teak quinone A) concentration was determined in the tissue


PIGMENT CONCENTRATION PER gm WET WEIGHT OF TISSUE
GROWTH OF TISSUE IN mg WET WEIGHT
AVERAGE WET WEIGHT OF CULLUS IN mg
mg CONCENTRATION OF PIGMENT TEAK QUINONE A / gm WET TISSUE
DAYS
2000
1600
1200
800
400
0
0·20
0·16
0·12
0·08
0·04
0
0
10
20
30
40
50
60
70

FIGURE–12

over a period of 0-70 days at 5 to 10 day intervals. The amount of pigment in the tissue during the first 30 days increases gradually (Fig. 12) and later shows a sharp rise, reaching its maximum on the 45th day. This corresponds to the end of the logarithmic phase of growth. After this period the pigment concentration gradually drops till the 70th day.

(b) Effect of gibberellic acid (GA_3)

Teak tissue was grown either with or without gibberellic acid and the pigment estimated in the tissue after 35 days growth (Table 36). Tissue grown with GA_3 contained three times more teak quinone A than tissue grown in its abscence.

The influence of gibberellic acid when it was autoclaved or sterilized by filtration is also shown in Table 36. When GA_3 was filtered and added aseptically the concentration of teak quinone A was doubled, but when autoclaved with the medium it was about seven times higher than the control. This indicates that during autoclaving the GA_3 possibly breaks down to products which enhance pigment production.

(c) Effect of different gibberellins

Since autoclaved GA_3 was found to have a marked positive effect on pigment production the different gibberellins were autoclaved with the medium for testing their effect on pigmentation in teak callus (Table 37). Pigment production on tissues grown with GA_4 was the maximum being 11 times more than the control, followed by GA_1, GA_{13}, GA_5, GA_7 and GA_3 where the levels were 4 to 5 times more.

Table - 36

Effect of gibberellic acid (autoclaved and aseptically added)

Media: Mineral salts - Murashige and Skoog's
Organic supplements - Murashige and Skoog's
Other supplements(ppm) IAA - 5; kinetin - 1; sucrose - 3%.

No.	Supplements	Teak quinone A per g. wet tissue
1	-	0.058
2	GA_3 10 ppm (autoclaved)	0.256
3	" (aseptically)	0.098

Table - 37

Effect of various gibberellins on pigment production

Media: As in Table 36 all gibberellins added at 10 ppm and autoclaved

No.	Supplements	Teak quinone A mg/gm of wet tissue
1	-	0.061
2	GA_1	0.287
3	GA_3	0.217
4	GA_4	0.706
5	GA_5	0.236
6	GA_7	0.231
7	$GA_4 + GA_7$	0.090
8	GA_9	0.089
9	GA_{13}	0.263

It is surprising that a mixture of GA_4 and GA_7 did not increase pigment formation.

The number of pigments as determined by PLC also varied with the different gibberellins. Tissues grown on GA_1, GA_7 or GA_{13} showed a similar type of pattern similar to that of the controls with a total of eight different bands. On GA_3, GA_4 or GA_5 the tissue extracts showed one extra violet coloured band situated above band.B. GA_4, GA_4/GA_7 and GA_9 grown callus were similar but had in addition one violet band above band A (i.e. teak quinone A).

(d) Effect of carbohydrates

While studying the effect of different carbohydrates on growth (Table 14) it was observed that pigmentation differed with respect to the carbon source supplied. Table 38 shows the effect of sucrose, glucose, galactose and fructose on pigment formation. Maximum pigment formation was observed with fructose followed by sucrose and galactose. With glucose, pigment formation was negligible. The bands observed on PLC with glucose, galactose and sucrose grown callus were similar while in fructose grown callus band C was very prominent, and a very dark violet streaking above the base line was also present. Although galactose was the best carbon source for growth, tissue grown on this carbon source contained almost the same amount of pigment per gram of tissue as sucrose grown tissue. The results suggest that the constitution and metabolsim of these different carbon sources by teak callus differs particularly with respect to pigment formation.

Table - 38

Effect of various carbohydrate sources on pigmentation

Media: Mineral salts - Murashige and Skoog's
Organic supplements - Murashige and Skoog's
Other supplements (ppm) - IAA - 5; kinetin - 1; all the carbohydrates were supplied at 3%

No.	Supplements	Teak quinone mg/g wet tissue
1	Sucrose	0.051
2	Glucose	0.003
3	Galactose	0.046
4	Fructose	0.143

Table - 39

Effect of various nitrogen sources on pigmentation
(Control contains macro salts as per Murashige & Skoog's medium)

Media:
Mineral salts - Murashige and Skoog without nitrogen sources
Organic supplements - Murashige and Skoog's
Other supplements (ppm) - IAA - 5; kinetin - 1; sucrose - 3%.
In experiments 2-5 inorganic nitrogen sources were omitted and N_1 sources added at 840 mg N_1/l. medium and KCl was used for supplying K^+ ions in experiments 3, 4 & 5. YE was added at 0.2%

No.	Supplements	Teak quinone A mg/g wet tissue
1	Control	0.059
2	KNO_3	0.257
3	NH_4NO_3	0.055
4	Allantoin	0.024
5	YE	0.579

(e) Effect of nitrogen sources

Pigment concentration in tissue grown on different nitrogen sources is indicated in Table 39. Highest pigmentation was observed with yeast extract grown tissue followed by potassium nitrate, ammonium nitrate and allantoin. Tissue grown on potassium nitrate imparted a red colour to the medium. The pH of this medium was found to be about 6.5 and may have been responsible for this colour. In the other cases the pH of the medium was unchanged.

(f) Effect of coconut milk and NAA

In all the previous experiments carried out to study pigment production with regard to different nutritional factors both kinetin and IAA were used together. Table 40 indicates the effect of CM and NAA on pigment production.

The callus tissue gave four times more teak quinone A when kinetin was replaced by coconut milk and gave almost half of it when IAA was replaced by NAA.

(g) Effect of growth retardant and GA_3

The effect of the growth retardants on pigmentation was studied in the presence and absence of gibberellic acid (1 ppm) (Table 41). A 40 fold increase in pigment formation with respect to teak quinone-A was observed with ABA 0.1 ppm. When GA_3 was added together with ABA (0.1 ppm) the amount of pigment formed was greatly reduced whereas when GA_3 was combined with ABA (0.01 ppm) pigment formation increased.

Table - 40

Effect of CM and NAA

Media: Mineral salts - Murashige and Skoog's

Organic supplements - Murashige and Skoog's

Other supplements (ppm) IAA - 5; NAA - 2; kinetin - 1; sucrose - 3%; CM - 15%.

No.	Supplements	Teak quinone A mg/g wet tissue
1	Kinetin, IAA	0.015
2	CM, IAA	0.060
3	Kinetin, NAA	0.007

175

Table - 41

Effect of growth retardants and GA_3 on pigmentation

Media: Mineral salts - Murashige and Skoog's
Organic supplements - Murashige and Skoog's
Other supplements(ppm) - IAA - 5; IAA - 1; ABA - 0.01 & 0.1; AMO 1618 - 0.1; CCC - 0.1; GA_3 - 1; sucrose - 3%.

The growth retardants were added aseptically to medium while GA_3 was autoclaved with the medium.

No.	Supplements	Teak quinone A mg/g wet tissue
1	-	0.015
2	GA_3 - 1 ppm	0.181
3	ABA - 0.01 ppm	0.033
4	ABA - 0.1 ppm	0.052
5	ABA - 0.01 + GA_3 1 ppm	0.275
6	ABA - 0.1 ppm + GA_3 1 ppm	0.135
7	AMO 1618 - 0.1 ppm	0.038
8	AMO - + GA_3 1 ppm	0.137
9	CCC - 0.1 ppm	0.032
10	CCC + GA_3 1 ppm	0.086

The pigment pattern as observed from PLC bands was different with the different growth retardants. The control, GA_3 (1 ppm) and ABA (0.01 and 0.1) grown callus showed 8 bands. AMO 1618, GA_3 + ABA (.01 ppm), GA_3 + ABA (0.1 ppm) and GA_3 + AMO 1618) grown callus gave in addition a violet band between teak quinone-A and band B whereas CCC grown callus showed a violet band after band B whereas and above band C, which was not observed when GA_3 was also added to this medium.

ABA (0.01 ppm), AMO 1618 (0.1 ppm) and CCC (0.1 ppm) gave two to two and half times increase in teak quinone-A. GA_3 reversed the effect of ABA on growth but pigmentation increased eight times when GA_3 (1 ppm) and ABA (0.01 ppm) were added together than with ABA (0.01 ppm) alone. In the case of ABA (0.1 ppm) and GA_3 (1 ppm) together, the teak quinone-A concentration was almost half that with ABA (0.1 ppm) alone. This shows that the synergic effect of GA_3 and ABA for pigment production depends on their respective concentrations. In case of AMO 1618 and CCC, GA_3 increased pigment concentration three fold and two and half fold compared to AMO 1618 and CCC alone but it did not enhance growth.

(h) Effect of light

The effect of light on pigment formation was tested on tissue grown on a CM containing medium when kinetin was omitted (Table 42). The tubes were illuminated with light from four fluorescent 40 watt day light tube lights situated about 15 cm

Table - 42

Media: Mineral salts - Murashige and Skoog's

Organic supplements - Murashige and Skoog's

Other supplements(ppm) - IAA - 5; CM - 15%, Sucrose - 3%.

No.	Conditions	Teak quinone A mg/g wet tissue
1	No light	0.060
2	16 hrs/day light	0.147
3	16 hrs/day light (Ist subculture)	0.159

from the tube for 16 hours. The tissues turned green within the first 10-20 days but later they became dark brown. Light grown tissue contained nearly double the amount of pigment. This tendency to produce higher levels of pigment in light was not lost even after subculture.

SECTION 2

Biogenesis of teak quinone - A

Experimental and Results

Tissue culture techniques are being used for the study of biogenesis of various plant products. Higuchi (1962), Fritig et al.(1970), Dougall (1965), Hosel, Shaw and Barz (1972), Austin and Brawn (1973), Chen, Stohs and Staba (1969) have studied synthesis and degradation of primary and secondary metabolites of plants by using tracer techniques in conjunction with tissue culture.

To study the biosynthesis of teak quinone-A, two methods for administering labelled compounds were employed (1) as in Higuchi's (1962) work on biosynthesis of lignin in white pine callus and (2) based on Fritig et al. (1970) work on biosynthesis of coumarins in tobacco callus culture.

Method I

In the previous section it was shown that pigment production increases rapidly between 30 to 45 days. Hence the labelled precursors were fed to 30 days old callus in its logarthmic phase of growth.

In the first method, tissues were starved for 24 h. by immersing them in salt solution (Murashige and Skoog's) at pH 5.0 to 6. The callus as transferred to the radioactive solution of 2-^{14}C acetate (specific activity 1.829 mC/m mole). The tissues were harvested after 24 h, washed free of labelled solution and dried. The callus as extracted as described in Chapter IV and teak quinone-A was isolated by preparative layer chromatography. Teak quinone-A

Table - 43

No.	Method followed	Precursor fed	Chromato-graphy No.	Sp. activity of the pig-ment in dpm/m mole	Sp. activity in mC/m mole	% incorporation
1	Method 1	2-^{14}C Na-acetate 1.82 mC/m mole	5th	3.78×10^{6}	0.175×10^{-2}	1.94×10^{-3}
2	Method 2 one day	2-^{14}C Na-acetate 4.667 mC/m mole	5th	9.2×10^{6}	0.417×10^{-2}	6.63×10^{-3}
3	Method 2 15 days feeding	2-^{14}C Na-acetate 4.667 mC/m mole	3rd	22.49×10^{6}	0.101×10^{-1}	7.24×10^{-2}
			4th	16.6×10^{6}	0.75×10^{-2}	5.6×10^{-2}
4			5th	17.8×10^{6}	0.80×10^{-2}	3.8×10^{-2}
4	Method 2 1 day feeding	2-^{14}C-MVA 5.85 mC/m mole	4th	9.88×10^{6}	0.44×10^{-2}	0.27×10^{-1}
			5th	9.88×10^{6}	0.44×10^{-2}	0.245×10^{-1}
5	Method 2 15 day feeding	2-^{14}C-MVA 5.85 mC/m mole	4th	31.6×10^{6}	0.14×10^{-1}	1.66×10^{-1}
			5th	27.2×10^{6}	0.12×10^{-1}	1.14×10^{-1}

showed incorporation of acetate but the percentage incorporation was found to be low compared to that obtained in the second method (Table 43).

Method II

In this method 2-^{14}C Na-acetate having specific activity of 4.665 mC/m mole was dissolved in water and made upto 1 ml with acetate buffer at pH 6.0; 0.05 ml. of labelled solution was placed over actively growing callus on Murashige and Skoog's medium on its 30th day. The solution was allowed to metabolise for one day and then the tissues were harvested, washed, dried and extracted. A higher percentage incorporation of 2-^{14}C Na-acetate into Teak-quinone-A was obtained by this method than by Method I (Table 43).

Thompson and Burnett and Thomson (1967) have shown that in short term tissue feeding of labelled mevalonate to Rubia tinctorum plants, equilibration was not reached and only the carboxyl group of pseudopurpurin got labelled. For the biogenesis of alizarin and other compounds in this plant they fed labelled mevalonate for fifteen days. Teak callus was fed by the second method with 2-^{14}C Na-acetate having a specific activity 4.667 mC/m mole for fifteen days. After that teak quinone-A was isolated and purified as usual. The percentage incorporation was better than in one day feeding experiments (Table 43; Expt. 3).

Incorporation of labelled mevalonate

A known amount of a benzene solution of 2-^{14}C mevalonic acid lactone was vacuum dried and then treated with a solution of potassium bicarbonate at 37° for one hour to convert it into the potassium salt.

Two experiments were carried out. In the first 2-^{14}C mevalonate having a specific activity of 5.85 mC/m mole was fed according to method II for one day. Teak quinone-A was isolated and purified according to the standard method and percentage incorporation of labelled precursor was calculated (Table 43; Expt. 4).

In a second experiment potassium salt of mevalonic acid having a specific activity of 5.85 mC/m mole was fed for fifteen days according to Method II and the percentage incorporation into teak quinone-A was calculated. It was observed that mevalonic acid had a higher percentage incorporation than acetate. It was also observed that mevalonic acid fed for fifteen days had a higher percentage incorporation than one day fed callus (Table 43; Expt. 5).

When acetate was fed for 15 days the bands above and below of teak quinone A were isolated by extraction with acetone and the radioactivity measured. As they showed a very low count after repeated chromatography, it was assumed that there was no impurity in teak quinone-A. In all the four experiments the counting for ^{14}C was done on a liquid scintillation counter using Bayer's solution. Teak quinone-A was dissolved in a known quantity of methanol taken for counting. The compound was purified till it gave a nearly constant specific activity (see table).

SECTION 3

DISCUSSION

The biosynthetic potentialities of plant tissue and organ cultures have been reviewed by various workers (Staba 1963; Krikorian and Steward 1969; Tomita 1971) and generally they differ from that of the intact plants. The isolation of teak quinone-A from teak callus cultures also supports this view, as this is the first time it has been found in nature and is not similar to any of the anthraquinones isolated from the teak tree (Thomson 1971a).

The effect of various factors like light, auxins, cytokinins, carbohydrates etc. on secondary plant metabolites have been studied by various workers. In the present chapter the effect of a few of these factors on the production mainly of teak quinone-A is described. Only those factors were studied where either growth was enhanced markedly or pigmentation visibly increased. In experiments where the concentration of teak quinone-A was higher, the number of spots in TLC plates was also found to be more, indicating possibly that a particular nutritional factor leads a greater influence on the biogenesis of these pigments.

(a) Age and pigmentation

Since the formation of lignins, flavonoids, anthraquinones, etc. is related to the process of ageing in plants, a relation between growth and pigment synthesis in teak callus was studied. An increase in pigment production with age of the callus has been shown with Jerusalem artichoke and Haplopappus gracilis (Ibrahim et al.

1971). Alkaloid production was also found to be increased with age of T. foenum-graecum cultures (Khanna and Jain 1972) or as the tissues differentiated (West and Mika 1957; Newmann 1968; Roddick and Butcher 1972).

Maximum pigment formation was observed with teak callus tissue just before the steady phase of growth. No cytological studies were carried out on teak callus to determine whether any cellular differentiation had taken place.

(b) Effect of gibberellic acid

The biological activities of gibberellic acid and other gibberellins in plants have been extensively reviewed (Paleg 1965; Lang 1970). Gibberellic acid (GA_3) was reported to have an effect on the flavanol content in Pisum sativum leaves (Moore and Pecket 1972) and also on the enzyme activities (Jacobsen, Scandialois and Warner 1970, Pillet 1957) and development of autumn colour and leaf fall in a few woody plants (Brain, Petty and Richmond 1959). Gibberellic acid has, however, been shown to enhance the uptake and incorporation of anthocyanin precursors by callus cells of Daucus carota (Louchele, Lehner and Sertz 1974). In the presence of autoclaved gibberellic acid teak callus showed a 3-fold increase in the amount of teak quinone-A in freshly isolated cultures, whereas the increase was over 7 fold with cultures which had undergone many subcultures. The increase in pigment production in the presence of aseptically added gibberellic acid was relatively less. Pryce (1973) recently has shown that on autoclaving, only

1-2% GA_3 remains intact, the rest decomposes mainly into 6 products. The increase in pigmentation of the cultures by autoclaved GA_3 may be due to one of these products or to all of them.

(c) Effect of various gibberellins

All the gibberellins tried were structurally quite different from each other and except for GA_4 which showed a maximum enhancement and $GA_4 + GA_7$ and GA_9, a minimum enhancement of pigmentation, all the other gibberellins tested gave pigmentation similar to or little more than GA_3. This result shows that GA_4 diverts the cell metabolism for production of more of teak quinone-A and also the number of pigments produced were more than with GA_3 grown callus.

(d) Effect of carbohydrates

There are very few reports on the effect of carbohydrates on secondary plant metabolites. Davydova and Butenko (1968) while studying lipid metabolism showed that the amount of linoleic acid and linolenic acids was increased by a higher sucrose concentration in the medium. Constabel (1968) has shown that tannin increased with higher concentrations of glucose, while Schantz, Duranton and Peyrier (1967) reported that total carotenoids decreased with increase in glucose concentration. The effect of different concentrations of sucrose was not studied, but using various carbohydrates (Table 38) it was observed that with fructose maximum pigment formation occurred. Parthenocissus (Bleichert & Ibrahim 1974) tissues have been reported to produce flavonoids only in the presence of 5% glucose. Fructose or sucrose were ineffective. Fructose was found to be effective for growth but not

for pigment production by poplar callus cultures (Matsumoto, Nishida, Noguchi and Tomaki 1973). This is contrary to the results obtained with teak callus.

(e) Effect of various nitrogen sources

The effect of various nitrogen sources on pigmentation has not been reported. In the present study, cultures grown with YE, a complex substance and potassium nitrate showed a 5 to 10 fold increase in pigmentation. This indicates that nitrogen metabolism also affects pigmentation.

(f) Effect of auxin and cytokinin

The effect of cytokinins and auxins on alkaloid, antocyanin and isoflavone production in tissue cultures has been reported. Konoshima et al. (1971) have reported an increase in nicotine production by kinetin and an inhibition by auxins with tobacco cultures. Recently it has been shown that the production of nicotine by tobacco callus cultures is very sensitive to auxin concentration, being higher at lower auxin concentration (Takahashi and Yamada 1973). Alkaloid formation by Tylophora indica cultures was not stimulated by auxins (Benjamin and Mulchandani 1973). Anthocyanin synthesis was found to be suppressed by auxin in Haplopappus gracilis (Stickland and Sutherland 1972a) callus cultures, while Jerusalem artichoke required NAA for anthocyanin formation (Ibrahim et al. 1971) although it grew on IAA or 2,4D. An auxin was also found to increase anthocyanin production with carrot (Sugano and Hayashi 1967), Haplopappus gracilis

(Blakely and Steward, 1961, 1962; Ardenne 1965) and cape marigold (Harborne, Arditti and Ball 1970). The results with teak callus indicate that IAA was better for pigmentation than NAA. The concentrations of NAA (2 ppm) and IAA (5 ppm) were however different. For the synthesis of the deoxyisoflavone, diadzin (Miller 1969) a zeatin cytokinin was not required if 2,4D was present in the medium, but kinetin did affect the synthesis of an unknown compound if 0.6M sucrose or mannitol was used instead of 0.1 M sucrose (Miller 1972). Formation of paniculide A, paniculide B and paniculide C by callus cultures of Andrographis paniculata was not affected by 2,4D, NAA, IAA, CM, kinetin or CH (Butcher and Connolly 1971). Even without any growth factors callus could synthesise these compounds. Coconut milk was found to induce pisatin formation in pea leaf discs (Bailey 1970). Konoshima et al. (1971) have shown that in their strain of tobacco callus kinetin induced nicotine formation while auxin inhibited it. For anthocyanin synthesis (Ibrahim et al. 1971) in Jerusalem artichoke and carrot callus, kinetin at low levels was essential while for rose and apple a high level was required. CM stimulated quinone formation in teak callus. Sairam and Khanna(1970), Khanna and Jain (1972), Khanna and Nag (1972) and Veliky (1972) have shown that small amount of precursor can stimulate synthesis of alkaloids in various cultures. CM contains shikimic acid which is a probable precursor of teak quinone-A. The increase in pigmentation of teak callus may be due to the various growth factors present in it.

(g) Effect of growth retardants

There are very few reports on the effect of growth retardants on pigmentation in tissue culture. ABA has been reported to reduce growth, lignin content, scopolin and scopoletin in tobacco tissue (Li et al. 1970). GA_3 overcame this ABA inhibition of growth and lignin synthesis and partially reversed scopoletin production. CCC with NAA increased carotenoids in tobacco cultures (Hardilik, Camburg, Nazareva 1973). B-995 did not influence the composition of carotenoids in a similar manner. With teak callus, quinone synthesis was found to be stimulated to a lesser degree with ABA (0.01 ppm), AMO 1618 (0.1 ppm) and CCC (0.1 ppm) and to a greater degree with ABA (0.1 ppm). With 0.1 ppm of CCC or AMO 1618 or ABA (0.01 ppm), GA_3 1 ppm increased pigmentation.

(h) Effect of light

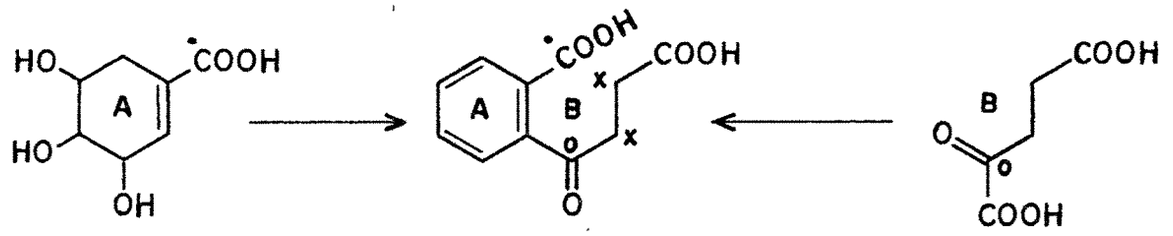
Callus cultures exposed to light showed differences in their biosynthetic capacity (Ibrahim et al. 1971; Brunet and Ibrahim 1973; Stickland and Sunderland 1972b). Phenolics in J. communis callus were depressed from 25 ± 4% in darkness to 12 ± 1% on illumination (Constabel 1963) although it did not affect growth. Lipids were increased when kalanchoe callus was illuminated (Thomas and Stobart 1970). Rutacultin a new coumarin was isolated from light grown suspension cultures of Ruta graveolens (Scharlemn 1972). Light stimulated synthesis of apigenin in soybean (Hahlbrock 1972) and inhibited polymerisation of leucoanthocyanins and increased all monomeric components in tea callus (Forrest 1969). Light has also been shown to affect anthocyanin and flavonoid synthesis in callus of poplar (Matsumoto et al 1973), Haplopappus gracilis (Stickland and Sunderland 1972b)

citrus peel (Brunet and Ibrahim 1973) and rose (Davies 1972). Anthycyanin synthesis was also shown to be promoted at different wavelengths of light (blue, white, red and green) with cultures (Matsumoto et al. 1973, Stickland and Sunderland 1972b). The results with teak callus also show a stimulation of pigment production in light (Table 42, 1, 2 & 3), which was not lost on subcultured light grown callus.

SECTION B (contd.)

In the present studies teak quinone-A isolated from callus culture of teak has been proved to be 1-methoxy, 2,5-dihydroxy, 3-methyl anthraquinone. As shown in Fig. I (Introduction) the various ways of folding of the polyketide chain leads to (1) emodin and endocivein, (2) solorinic acid and versicolonin-A, etc. In all these cases the hydroxyl groups are located at 1,3,6 & 8-position of anthraquinones. The variation in the basic structure results from o-methylation, side chain oxidation, chlorination, dimerisation and the introduction or elimination of nuclear hydroxyl groups (Thomson 1971). It is known that removal of hydroxyl groups can take place before aromatization of the polyketide chain. For the formation of teak quinone-A hydroxylation at 5-position has to take place, which can occur in the presence of hydroxyl at 6- or 8- position. It means that if teak-quinone A is formed by condensation of polyketide chain there should be the presence of two hydroxyl groups in ring A at positions 5, 6- or 5,8. Juglone (Leistner and Zenk 1968d) (5-hydroxynaphthaquinone) has been found to incorporate carboxylic group labelled shikimic acid and it was observed that keto-C-atoms were equally labelled. Degradation of ring-A of the juglone molecule labelled from 1,2-^{14}C shikimate showed that activity is symmetrically distributed in the molecule (Leistner and Zenk 1968d), and this finding was also confirmed by Leduc, Donsette and Azerad (1970). This suggests that possibly 1,4-naphthaquinone is an intermediate in juglone synthesis.

SCHEME - II

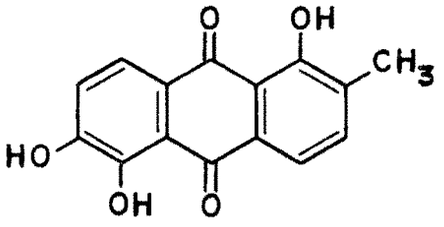


SHIKIMIC ACID
XLIV

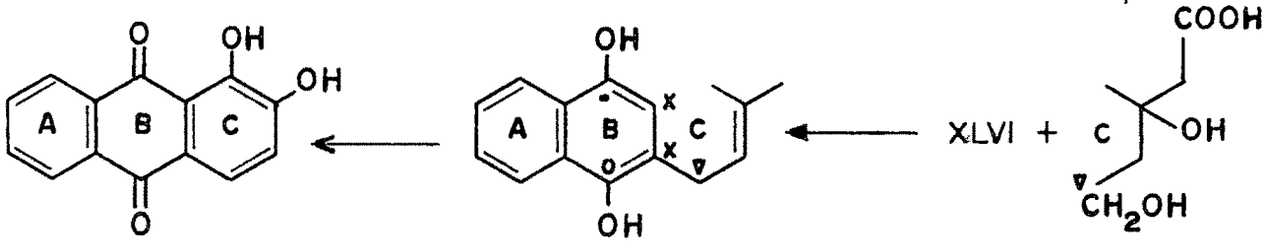
O-SUCCINYL BENZOIC ACID
XLVI

α-KETOGLUTARATE
XLV

NAPHTHALENE OF
UNKNOWN STRUCTURE +
γ-γ-DIMETHYL PP



MORINDONE
XLIX



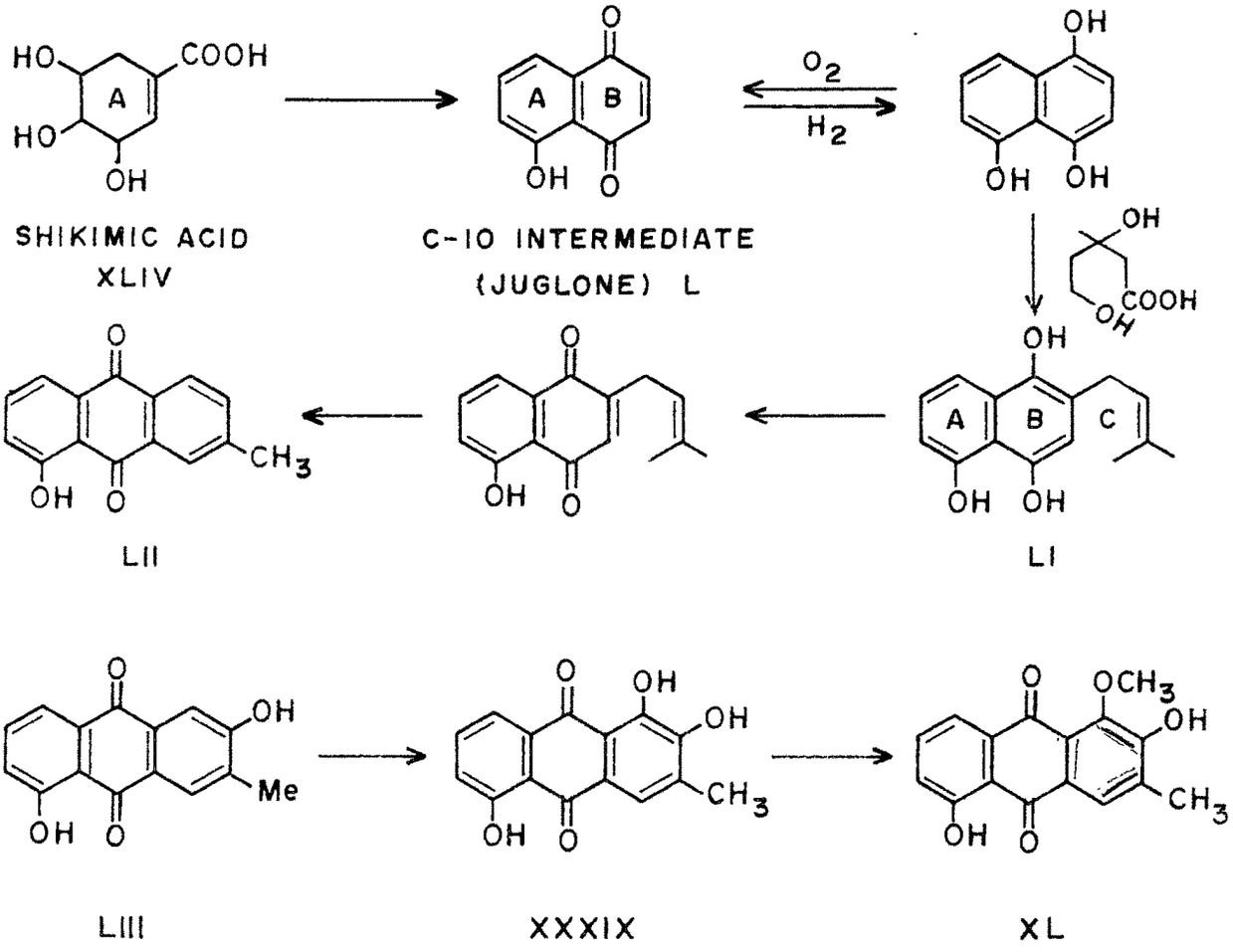
ALIZARIN
IX

2-(γ-γ-DIMETHYLALLYL)
-NAPHTHOQUINOL
XLVIII

XLVII

BIOGENESIS OF ALIZARIN (LEISTNER, 1973, a b)

SCHEME III



BIOGENESIS OF TEAK QUINONE-A

(1957)
Sandermann and Deitrichs suggested that naphthaquinones might be precursors of methyl anthraquinones in teak as both of them occur together. From the above studies it can be speculated that teak tissue synthesised teak-A via the route shown in Scheme-II.

For the confirmation of the above hypothesis teak-callus cultures were fed with ^{14}C labelled precursors. As described in Section 2, two methods were employed. From table 43 it can be seen that method-II was better than method-I. This may be due to the inability of teak callus to grow in liquid medium. The percentage incorporation of 2-^{14}C-Na-acetate by method II is almost 3 times more than by method I. When mevalonate was fed for one day by method II, the percentage incorporation was 0.024, but with Na-acetate it was 0.0066, indicating that mevalonic acid is the immediate precursor for teak quinone-A. Both the precursors were allowed to be metabolised for fifteen days separately and the percentage incorporations of acetate and mevalonate were calculated (see Materials and Methods). In this case also mevalonate (0.11%) incorporation was three times more than acetate incorporation(0.038%).

The incorporation of acetate to such a high percentage in 15 days feeding experiments can be explained on the basis of Listner's (1973b) results with Madder root for alizarin biosynthesis. According to these results C-10, C-13 and C-14 of the alizarin molecule come from α-ketoglutaric acid which in turn is derived from Kreb's cycle, which utilizes acetic acid as acetyl CoA. Recently Listner (1973a) has proved shikimate origin of morindone (1,5,6-trihydroxy 2-methyl

anthraquinone) via O-succinoyl benzoic acid. Scheme II shows the biogenesis of alizarin and morindone as proved by Leistner (1973a and 1973b). From the data obtained on percentage incorporation of acetate and mevalonate into teak quinone -A in teak callus culture (Listner 1973a, 1973b),(Leistner and Zenk 1968a, 1968b, 1968c, 1968d) and results with alizarin and juglone biosynthesis it seems possible that teak quinone-A is synthesised via the route postulated in Scheme III.

Due to the very low yield of this compound from tissue culture and also the limited availability of labelled precursor it was not possible to study degradation of labelled teak quinone-A and establish the position of labelled-C in the anthraquinone molecule.

SUMMARY AND CONCLUSION

***

SUMMARY AND CONCLUSIONS

I. The initiation and maintenance of callus cultures from teak, jack, mulberry and poplar

The conditions for initiation and maintenance of callus cultures from branch or seedlings of *Tectona grandis* (teak), *Artocarpus heterophyllus* (jack), *Morus alba* (mulberry), and *Populus nigra* (Poplar), were determined. These cultures were maintained *in vitro* for over six years by subculture every 30-35 days and may be regarded as viable cultures of these trees. The cultures from teak, mulberry and jack represent the first viable cultures obtained from these trees.

For initiation of callus from explants in teak, mulberry, jack and poplar, the minimum requirements are agar medium containing mineral salts, vitamins, glycine, and kinetin or coconut milk.

Pantothenate and biotin enhanced callus formation from explants of teak, mulberry and poplar, but not from jack. The latter was stimulated by malt extract and yeast extract, whereas initiation of teak callus was initiated by malt extract.

Benzothiazole-2-oxyacetic acid was the best auxin for callus initiation from jack explants, and IAA or NAA or 2,4D for explants from the other trees. Kinetin or coconut milk was essential for callus formation in teak explants.

The basal media used for initiation of callus from teak and jack explants did not support growth of these cultures on subculture. In the case of mulberry, two auxins 2,4D and IAA, were added to the medium for the initiation of callus, but IAA was sufficient for the maintenance of the culture. Poplar could be maintained on the same medium on which callus was initiated from the explants.

For maintenance of viable callus cultures of teak, high salts medium containing vitamins, glycine, casein hydrolysate, IAA and kinetin was the best, while mulberry was grown on high salt medium containing vitamins, glycine, IAA, kinetin, pantothenate and biotin. Jack callus was maintained on Blaydes' medium and poplar on Murashige and Skoog's salt solution containing vitamins, glycine, kinetin, NAA, pantothenate and biotin. 2,4D was the best auxin for jack, whereas poplar was grown on NAA.

Jack callus had a specific temperature requirement of 30 ^{o}C and did not survive at 26 ^{o}C, the temperature at which the other tissues were maintained.

All the cultures except teak were soft and friable. Teak was hard and compact having a yellow to orange colour. Initially mulberry cultures were a mixture of callus and roots. This capacity to form roots was lost after about twenty subcultures. Mulberry and jack were light brown in colour, while poplar callus, which was initially white in colour, turned light brown after four years of maintenance in in vitro.

II. Nutritional studies on teak callus

Detailed studies were undertaken on the nutrition of teak callus. Murashige and Skoog's mineral salts were better than media with low salt concentrations which were adequate for callus initiation from explants.

Ammonium nitrate, potassium nitrate and yeast extract were good nitrogen sources.

The callus could utilize a variety of carbohydrates, like glucose, maltose, fructose and sucrose, but the best carbon source was galactose.

Teak callus had an absolute requirement for inositol and the optimum concentration was 100 ppm. Pyridoxine added alone or together with thiamine in the presence of IAA, kinetin, inositol and glycine, greatly stimulated growth. Nicotinic acid was not essential for growth.

An auxin was essential for continuous growth of teak callus culture. IAA was the most effective auxin and its optimum concentration was between 5-10 ppm.

Kinetin stimulated the growth of teak culture even at levels of 0.1 ppm. With coconut milk the growth was twice as much as with kinetin. Tissue grown on media containing either diphenyl urea, zeatin or thiourea gradually increased in weight with subculture, possibly due to an adaptation of the tissue to these growth factors.

Gibberellic acid had a stimulatory effect on fresh isolates of teak but later this effect was not observed. Of various gibberellins tried, GA_7 was inhibitory.

Growth of teak cultures was enhanced by adenine and uracil, while cytidylic acid inhibited it.

Even at low levels abscisic acid, AMO 1618 and CCC were inhibitory. The abscisic acid inhibition was overcome by GA_3.

Growth of teak callus was better at 30°C than at lower temperatures.

The optimum agar concentration was 0.7%.

III. Quinones from teak callus

Three anthraquinones were isolated and identified from acetone extract of teak callus. One of the compounds was 1,2,5-trihydroxy,3-methylanthraquinone identified by chromatography with a synthetic compound.

The second compound, teak quinone B_1, was digitolutein (1-methoxy,2-hydroxy,3-methylanthraquinone). This compound was also shown to be present in teak wood extract. This is the first time that digitolutein has been shown to be present in teak wood and teak callus.

Teak quinone A, a new anthraquinone was isolated and its structure was shown to be 1-methoxy,2,5-dihydroxy,3-methyl-

anthraquinone by using mass spectra, NMR, IR and UV. The structure was confirmed by synthesising its methyl ether from 3-methyl alizarin.

IV. Effect of different factors on pigmentation

Teak callus for the first few subcultures showed the presence of six coloured compounds on preparative layer chromatography. After prolonged subculture in in vitro, it showed the presence of two more compounds.

GA_3 increased the synthesis of teak quinone A. Autoclaved GA_3 gave more pigment than aseptically added hormone.

As GA_3 increased pigmentation, effect of GA_1, GA_4, GA_5, GA_7, GA_9, GA_{13} and $GA_4 + GA_7$ was studied.

With GA_4 maximum synthesis of teak quinone A was observed. The number of pigments produced was different with different gibberellins. Four types of chromatographic patterns were found with (a) GA_1, GA_7 and GA_{13}, which gave similar pattern to that containing no gibberellins; (b) GA_3 and GA_5; (c) $GA_4 + GA_7$ & GA_9; and (d) GA_4. This may indicate that different gibberellins direct the metabolism of pigment synthesis in different ways.

Although galactose was a superior carbon source for growth it did not enhance pigmentation. Fructose was the best carbon source for pigmentation and it increased the concentration of teak quinone A.

Yeast extract was superior to KNO_3, NH_4NO_3 or a combination of both the substances for pigment production.

Coconut milk was better than kinetin and IAA better than NAA for pigment production.

Abscisic acid, AMO 1618 and CCC increased pigmentation. The concentration of abscisic acid and GA_3 had a marked effect on the formation of teak quinone A. ABA at 0.1 ppm gave maximum formation of teak quinone A. When GA_3 at 1 ppm was added together with ABA at 0.1 ppm, the amount of teak quinone A was markedly reduced. However, GA_3 combined with ABA at 0.01 ppm increased pigment concentration considerably compared to ABA (0.01 ppm) alone.

Light stimulated teak quinone A synthesis.

V. Biosynthesis of teak quinone A

To study the biogenesis of teak quinone A, 2-^{14}C-acetate and 2-^{14}C-mevalonate were fed to actively growing teak callus.

From the percentage incorporation of 2-^{14}C mevalonate and 2-^{14}C acetate, it was concluded that probably ring C of teak quinone A was derived from mevalonate while C-10, C-13 and C-14 were derived from acetate.