METABOLISM OF PLANT CELLS GROWN IN VITRO
(STUDIES ON MAIZE, WHEAT, RICE AND SORGHUM)

A
THESIS
SUBMITTED TO
THE UNIVERSITY OF POONA

FOR
THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

BY

A. F. M A S C A R E N H A S, M.Sc.

Division of Biochemistry
National Chemical Laboratory,
Poona 8

1971

G E N E R A L C O N T E N T S

ACKNOWLEDGEMENT

The author wishes to record his 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 him by his colleagues Mr. R. R. Hendre, Mr. B. Seetharama Rao and Miss Meera Pathak during the course of this work. Thanks are also due to Mrs. Bathsion D. Benjamin for help rendered during the early stages of this work to Dr. C. SivaRaman for going through the manuscript and making valuable suggestions.

The author is indebted to the Director, National Chemical Laboratory for permission to submit this work as a thesis for the degree of Ph.D.

LIST OF ABBREVIATIONS

Abscisic acid	3-Methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl-2'-cyclohexene-1'-yl)-*cis*,*trans*-2,4-pentadienoic acid
AMO-1618	4-Hydroxyl-5-isopropyl-2-methyl phenyl trimethyl ammonium chloride,1-piperidine carboxylate
2-BTOA	Benzothiazole-2-oxyacetic acid
CCC	(2-Chloroethyl)trimethyl ammonium chloride
CH	Casein hydrolyzate (Casamino acids)
CM	Coconut milk
CMP	Cytosine mono phosphate
CSL	Corn steep liquor
2,4-D	2,4-Dichlorophenoxyacetic acid
DPU	*sym*-Diphenylurea
DNA	Deoxy ribonucleic acid
Edamin	Edamin-S
EDTA	Ethylene diamine tetraacetic acid
GA	Gibberellic acid (GA_3)
IAA	3-Indole acetic acid
IBA	3-Indole butyric acid
IPA	3-Indole propionic acid
Inositol	*myo*-Inositol
Kinetin	6-Furfuryl amino purine
ME	Malt extract
NAA	α-Naphthalene acetic acid
P_i	Inorganic phosphorus
RNA	Ribonucleic acid
2,4,5-T	2,4,5-Trichloro phenoxy acetic acid
YE	Yeast extract
Zeatin	6-(4-Hydroxy-3-methyl but-*trans*-2 enyl) amino purine

g	gram
l	litre
mg	milligram
OD	Optical density
µg	microgram
ppm	parts per million
psi	pounds per square inch
rpm	revolutions per minute

BM	Basal medium see Table 2D. page 48 for composition
BM_1	"
BM^1	"
BM^2	"
BM^3	"
BM_2P	"
WB	"
WB_1	"
WB^1	"
WB^2	"
WB_3	"

INTRODUCTION

Pages 1 - 38

C O N T E N T S

GENERAL INTRODUCTION

Tissues from a large number of dicotyledonous plants grown *in vitro*, but very few monocotyledonous plants have been grown in tissue culture. Only limited progress has been made in culturing plant tissues belonging to the latter group and in studying their nutrition, metabolism and differentiation. Studies on cell and tissue cultures of these important crop plants are potentially of value in crop improvement (319).

The present work deals with tissue cultures of maize, wheat, rice and sorghum, which are viable *in vitro* on prolonged subculture. The initiation of these cultures, their nutritional requirements and uptake of nutrients, their growth and differentiation in agar and liquid media and the chemical analysis and enzyme content of callus and root cultures form the subject of this thesis.

S E C T I O N 1

Scope of the literature survey

Earlier literature in the field of tissue culture of monocotyledonous plants, especially on cultures from root and stem tissues of cereals, which were shown to be viable on subculture, will be reviewed in the Introduction. No attempt will be made to survey the voluminous literature on plant tissue cultures which has extensively been reviewed (35,48,89,135,287,298,302,317,355) except for brief references to some of the important findings in this field.

SECTION 2

Historical

The experimental method known as "Tissue Culture" has been described by the late Professor P. R. White as "a way of reducing organisms to their lowest common denominator, the cell". "In tissue culture we take the organism apart, without ideally impairing the functions of the parts. We perform a delicate sort of vivisection, which does not 'hurt' the part we are interested in, which in fact permits it to continue to function far beyond the usual span of life". "Once isolated by this method we can study the cell or tissue or organ at leisure, look at it from all sides, and by many methods, subject it to experimental interventions, and then, if we wish, put it back into the organism, or put organs or cells of the same or of different organisms together in new ways for study" (356).

The aseptic culture of plant cells and tissues as a technique is now well established. The botanist Gottlieb Haberlandt clearly set forth the purposes and potentialities of cell culture, and though he was not successful in his attempts at plant tissue culture, he foresaw the use of cell culture as an elegant means of studying physiological and morphological problems (97,133). His failure was probably due to several reasons: the inadequacy of the nutrients, the use of mature cells and highly differentiated tissues especially from monocots which are generally difficult to grow.

In 1922 Robbins (239) and Kotte (132) reported the culture of excised plant roots. These workers were able to culture roots for several weeks but not for an indefinite period. Later investigations by White (349) on tomato roots firmly established the technique of continuous root culture and outlined the nutritional requirements for

sustained growth. In 1934 Gautheret (80) also published the first of his papers on cambial tissue cultures. In 1939 White (351), Gautheret (82) and Nobecourt (212) independently reported the unlimited culture of callus tissue. Thus for the first time the prolonged culture of unorganised plant cells or callus was achieved. (The terms "unlimited culture", "continuous culture", "prolonged culture", "indefinite growth", "successful culture" are used to describe plant cells or tissues which do not die or show diminution in growth rate on subculture and are truly viable when maintained in vitro in the same manner as bacteria or fungi. This thesis deals with such plant cultures and not with short-lived cultures.) The major factors contributing towards the success of these workers were probably the selection of a favourable type of tissue and favourable medium. After these pioneering studies, there has been rapid development in the field of plant tissue culture and a large number of plant tissues were successfully grown in vitro. The nutritional and other conditions for their growth were defined and the technique has found wide application in studies of plant physiology and pathology.

SECTION 3

Nutrition

After the successful establishment of plant tissue cultures detailed studies were made of the nutrition of plant cells grown *in vitro*. The discovery of vitamins and plant hormones was of importance in defining the nutrient requirements of these cultures. The media consisted of inorganic salts, a carbohydrate source, an organic nitrogen source and growth regulating substances such as auxins and cytokinins, and in some cases complex supplements (eg. coconut milk). Only a very brief outline of the vast literature on this subject will be given here. The nutrition of monocots will be dealt with in a later section.

(a) Inorganic salts and nitrogen

The inorganic salt solutions used by Gautheret (81) and White (353) in their pioneering work were later modified by a number of workers, who demonstrated that the inorganic salt solutions of White and Gautheret were not suitable or were suboptimum for obtaining continuous callus cultures of some tissues (45, 103, 110, 193, 302). With some of these media, enhanced growth of a number of tissues was obtained. These media generally contain higher levels of potassium, nitrogen and phosphorus. Callus cultures, like plants, require nitrogen, potassium, calcium, magnesium, phosphorus and sulphur and also iron, manganese, boron, zinc, molybdenum, copper and possibly iodine in trace amounts (103, 302). The nitrogen source for plant cultures is generally nitrate, though amino acid mixtures, urea etc. have also been used, generally as supplements to a nitrate containing medium. Heller (103), Riker and Gutsche (235) reported that their callus cultures required nitrate and could not utilize nitrites or ammonium salts as sole sources of nitrogen. Amino acid mixtures or individual amino acids were

ineffective as sole sources of nitrogen, but were stimulatory in the presence of nitrate for several plant cultures (193, 209, 223, 246).

(b) Carbohydrate sources

The ability of different sugars to support growth has been studied for a wide variety of tissues and it has generally been found that most cultures grow best on sucrose, glucose or fructose, this being the order of their effectiveness as carbon sources (83, 107, 108, 199).

(c) Vitamins

Most media formulations include only two or three vitamins, thiamine, niacin and pyridoxine (103, 355). Some however contain a large number of vitamins (thiamine, pantothenic acid, biotin, riboflavin, pyridoxine, para amino benzoic acid, niacin, choline, folic acid, ascorbic acid and vitamin B_{12}) (106, 117, 234). After the discovery of inositol as a constituent of coconut milk (227) it has been shown to be an essential nutrient or growth stimulant for several plant cultures (236, 362). However, it should be noted that there is no rigorous experimental proof in the case of most tissues that all the vitamins added are essential for growth.

(d) Growth regulating substances

Auxins: Gautheret (88) reviewed the work on the responses of callus cultures to auxin and concluded that while some tissues can be cultured without auxins (mostly crown gall tissues) others require them. The media employed for successful continuous culture of normal tissues usually contain one of the auxins, IAA, NAA or 2,4-D.

Kinins: The discovery of kinetin (6-furfurylamino purine) (176) and its isolation from aged or autoclaved herring sperm DNA was a major

development in the field of plant cell nutrition. Kinetin was shown to have a wide range of effects on plant cells which include cell division and organ initiation (262). It has been shown to enhance the growth of a number of callus cultures (255, 262). Cytokinins have been shown to be present in a number of plant materials such as transfer RNA of wheat germ and other tissues (46), water melon juice (155,156) etc. Letham (146) isolated from immature seeds of Zea mays a crystalline material, zeatin, with a very high cytokinin activity. It was shown to be 6-(4 hydroxy-3-methyl but -2 enyl) amino purine (148).

Gibberellins: Following the discovery of natural gibberellins in the tissues of higher plants (36) their effects on the growth of several cultured tissues was tested (19, 105, 193). In some cases it was stimulatory whereas in others it had no effect. Though an absolute requirement of an auxin or kinetin has been demonstrated for many plant tissues, an absolute requirement for a gibberellin has not been demonstrated either because it is not required or because it is synthesized in adequate amounts by the plant tissues.

Other growth factors: van Overbeek et al (218) first used coconut milk in culture media for the growth of Datura embryos. It has subsequently been widely used and has proved effective in supporting the growth of many tissues (8, 50). An analysis of coconut milk showed that it provides a variety of factors concerned with growth stimulation of plant tissue cultures (282, 329).

Other complex materials which have been used for the culture of some tissues are yeast extract (120, 321, 325), malt extract (118,151, 230).

S E C T I O N 4

Types of cultures

Tissue cultures are of many types. Tulecke (326) classifies them into three main groups: (1) Organ cultures such as flowers, roots etc. (2) Initial explants, which are special types of tissue , often aseptically excised from the parent organ, but not grown in continuous culture. (3) Tissue cultures, which are continuously cultured tissues derived from a specific plant part. (In most cases callus tissues induced by growth regulators as well as callus tissues obtained from crown galls or virus tumors will also be included in this group.) White (356) in his classification does not include short term cultures as in Tulecke's classification (326) but includes another group - suspension cultures in which there is ideally no organization, but in which, as in callus cultures, differentiation can be induced under special conditions. Cultures from single cells or clones can also be included in this group.

This thesis deals mainly with tissue cultures which are capable of contimuous growth in vitro on solid or in liquid media.

SECTION 5

Applications

Some of the interesting avenues opened up by plant tissue cultures are investigations relating to nutrition, metabolism, morphogenesis, pathology and genetics.

(a) Cell nutrition

Several tissues can be successfully cultured on chemically defined media. Since in tissue culture sterile conditions are used, it is possible to define the special nutritional requirements of any organ or cell. Krikorian and Berquam (133) state that though it is possible to culture many tissues, it is still very difficult or impossible to obtain a "best medium" as it is a major problem to study all the interactions of the separate components of such a system (organic and inorganic). The cultivation of single isolated cells has also been achieved in different ways (20,69,188,279,316); such as the addition of "conditioned" medium or the use of a "nurse tissue". However it is generally difficult to grow single cells of plants whereas large inocula can be grown. Whether the single cells have more complex nutritional requirements is not known and the elucidation of the growth requirements of single cells of plants remains a major problem of plant tissue culture.

(b) Metabolic studies

Metabolic studies have been carried out both in static and in suspension cultures. Alkaloids (12,29), amino acids (251,346), antibiotics (128,164), glycosides (43,247,248), steroids (18) are some of the many products isolated from plant tissue cultures. A number of enzymes have also been reported to be present in plant tissue cultures (92,135,222). Similar products have also been isolated using suspension cultures (269, 270).

(c) Control of growth and differentiation

An important problem in biology is the mechanism of differentiation. Plant tissue cultures have proved a valuable tool in such studies and have shown the important role of auxin-kinin balance in morphogenesis (75,165, 229,262). "The capacity to produce the plant body does not, however, reside in the zygote alone - indeed in the light of recent work it may well persist, even though suppressed, in almost anyliving cell of the plant body ..... cells which have passed through many cell generations in culture may still retain a degree of totipotency which is comparable with that of the zygote" (277). Steward et al (278) obtained whole plants from carrot cell suspensions. Embryogenesis of endive and other plant tissues (99,100,334) and haploid and tetraploid plants from cell cultures have also been reported (192).

(d) Pathology

Plant cultures have been of value in the study of plant tumors, the growth of plant pathogens and other problems related to plant pathology. White and Braun (357) showed that the continued presence of bacteria is not required for tumour growth and that Agrobacterium tumefaciens only triggers the formation of malignant cells. Morel (182) infected grape callus with grape mildew and Tiwari and Arya (315) grew the fungus Sclerospora graminicola on Pennisetum cultures. The utilization of tissue cultures for the production of virus free plants is of importance (62,178,186,233).

(e) Genetic studies

Torrey (318) showed that continued propagation in vitro produced changes in the chromosome number of cell populations. Similar chromosomal aberrations were also observed in Haplopappus gracilis suspension cultures (179,180). The growth of clones of cells of higher plants is

likely to be of importance in genetic investigations on somatic plant cells. Nitsch and Nitsch (211) obtained haploid plants of tobacco by culture of the anthers from which by suitable treatments they later obtained diploid plants (210). Sunderland and Wicks (305) obtained haploid tobacco plants from tobacco pollen and Niizeki and Oono (205) induced haploid rice plants from anther culture.

After this necessarily brief general outline of plant tissue culture, the literature on the culture of monocotyledonous plant cells and especially of cereals will be reviewed in the subsequent sections.

S E C T I O N 6

Tissue cultures of monocots

Though a large number of dicotyledonous plant tissues have been obtained as viable cultures, relatively few monocotyledonous tissues have been grown continuously in vitro. In his monograph on plant tissue culture published in 1959, Gautheret (89) listed 82 cultures of dicotyledonous plants and only 12 from monocots and of these published data were available only for 3. During the last ten years the number of dicot tissue cultures has increased considerably but very few of monocots have been described. The work on monocot cultures may be grouped into - 1) Root and stem cultures, 2) Endosperm callus culture, 3) Callus cultures from root and stem and 4) Embryo cultures. No attempt will be made to review the vast literature on the growth of excised immature embryos which is outside the scope of this thesis but will be limited to the other three types of cultures.

a) Root cultures

Root tips from maize and wheat seedlings were used in the pioneering attempts to grow excised roots by Kotte, Robbins and White (132,239, 348). These cereal tips showed a high initial rate of growth on transfer to the culture medium, but growth declined sharply after several days and the newly formed root axis progressively decreased in diameter. Fiedler (72) found that when excised maize roots were cultured in liquid media they sank to the bottom, exhibited a well developed cortex and became thicker. These early attempts were unsuccessful and the tissue failed to survive on subculture.

Maize

In 1940 McClary (167) reported the growth of excised maize roots through at least 18 transfers without progressive decline in their growth

rate, on a mineral salt medium containing 5 per cent glucose. However, later attempts by Bonner and Bonner (33) and by Robbins (240) to repeat the work of McClary proved unsuccessful. In 1950 Kandler (122) attempted to culture roots of different plants, but sterile cultures of maize roots could not be maintained *in vitro* for long. Pilet and Bonhote (225) attempted to grow excised roots of maize in liquid medium in the presence of IAA. Gibberellic acid at 10^{-5}M increased the length and number of roots, but the tissues did not survive on subculture. The successful growth of maize roots has not been reported except for the work described in this thesis.

Wheat

Burstrom (44) reported good growth of isolated wheat roots when using a commercial yeast extract called Cenovis but the roots did not survive on subculture. After extensive studies on isolated wheat roots Almestrand (5) reported that pyridoxine had a marked effect on cell division of this species, although it had no effect on the growth of isolated roots of rye. The first successful culture of excised wheat roots was obtained by Ferguson in 1963 (70). The medium used was White's (354) with 2 per cent glucose, about 2 ppm L-tryptophan, thiamine, pyridoxine and niacin and the wheat variety was Hilgendorf 61. Ferguson (71) later showed that the only vitamin that was necessary was thiamine. The medium finally used was White's (354) medium with glucose, thiamine and tryptophan or an auxin.

In 1961 Street *et al* (301) studied the effects of acid hydrolyzed casein and illumination on excised wheat roots, variety Elite 56. These factors did not prevent a marked decline in growth rate after 14 days of culture. Sutton *et al* (306) using excised wheat roots of the same variety found that autoclaved tryptophan markedly enhanced the growth of these

roots either singly or in the presence of a mixture of amino acids. In light, tryptophan was inhibitory to main axis growth, but enhanced lateral initiation in both light and dark cultures. Unheated tryptophan, IAA and indole acetonitrile did not have the growth effects of autoclaved tryptophan. Scott et al (250) found a marked increase in the percentage dry matter and robustness of excised wheat roots at a light intensity of 300 lux or more. In 1963 Carter and Street (57) found that autoclaved tryptophan at 25 to 50 ppm depending on the glucose concentration enhanced the lateral number, length and dry weight of excised wheat roots grown in the dark. Unheated tryptophan or IAA was inactive or of low and variable activity. However, IAA at an appropriate concentration (0.025 to 0.05 ppm) plus unheated tryptophan (50 ppm) could reproduce the growth promoting effects of autoclaved tryptophan. Derbyshire and Street (65) cultured excised wheat roots (Elite 56) in darkness on a modified White's medium containing 2 per cent glucose and found that increase in dry weight virtually ceased by the fifth day of incubation. In nitrate media, light initially retarded but later enhanced the production of new cells at the apical meristem. Light also promoted both the absorption of nitrate from the culture medium and the conversion of soluble organic nitrogen to protein nitrogen.

In 1962 Gudjonedotter and Burstrom (96) showed that excised wheat roots grown in light and in the presence of iron showed a marked inhibition of cell elongation. This light inhibition could be reversed by any one of six low molecular weight primary alcohols if added at concentrations of 10^{-2} or 10^{-3}M. Using excised roots of Eroica wheat seedlings Bjorn et al (26) found an inhibiting effect of light on their growth. The spectrum for the inhibition of elongation had 2 peaks of approximately the same height near 430 and 650 mμ. In 1965 Bjorn (25) found that when the excised roots of wheat, "Starke" were irradiated with

red light only traces of chlorophyll were formed. When blue light was used increased accumulation of chlorophyll a and b occurred. Irradiation with red light before or after irradiation with blue light strongly enhanced chlorophyll formation compared with controls receiving only blue light.

Talbot and Street (308) first observed that aeration of the root culture medium with air containing 5 or 10 per cent carbon dioxide enhanced growth, particularly lateral initiation, compared with aeration with air or carbon dioxide-free air. The beneficial effect of enhanced carbon dioxide pressure on the growth of cultured wheat roots could also be demonstrated in the presence of a high (40 per cent) and inhibiting level of oxygen. Later Talbot and Street (309) presented evidence that the growth of cultured wheat roots is enhanced in closed cultured systems not only by carbon dioxide but by other volatile products of root metabolism.

Fujiwara and Ojima (76) and Ojima and Fujiwara (215,216,217) also studied the growth of excised roots of wheat variety Norin No.55 on a culture medium supplemented with peptone. The roots did not survive many subcultures but had the unusual property of being able to grow to 1000 mm in 3 months in the first passage in a small volume of medium. Peptone was later replaced by tryptophan or IAA in light grown cultures.

Rye

Roberts and Street (238) were the first to report that clones of excised roots of Petkus II winter rye could be established in continuous culture from 10 per cent of the seedlings of their grain sample. They considered that their successful establishment of rye root clones followed from the addition of yeast extract or tryptophan to their

standard root culture medium, and the adoption of an appropriate subculture technique. The importance of the duration of growth intervening between successive subcultures and the length of the root tips transferred at each subculture suggested to them that a nutritive interrelationship between the meristematic and mature tissues might be important for the continuation of cell divisions in excised root tips. In 1957 Almestrand (7) successfully repeated the work of Roberts and Street. He found that rye roots could also be grown on a basic medium supplemented with glucose and 5 ppm of a commercial yeast extract in single root cultures. Subcultures were performed once a week by excising the main tip or a lateral. A certain amount of selection in the material took place. Some roots became thin as the cell division stopped and the elongation continued. The other types of roots retained their larger diameter, continued to grow and were subcultured for more than half a year. Casein hydrolyzate in concentrations upto 1000 ppm was beneficial for the cereal roots.

Barley and Oats

Almestrand (3,4,7) noted that he had difficulty in obtaining continuous growth of isolated roots of barley and oats. Continuous growth was not obtained as no new divisions took place in the meristem cells though cell elongation went on normally.

(b) Non-cereal cultures

Onion

Krikorian and Katz (134) obtained cultures of onion root tips Allium cepa (Trapp's strain of Downing yellow globe) and maintained them for a period of over three years on White's basal medium (355) supplemented with coconut milk, inositol, casein hydrolyzate and 2,4-D.

They found that a minimal length of 3 mm was needed although cultures could be established in some cases even with 1 mm root tips.

(c) Stem tip cultures

In addition to excised root tips, excised stem tips have also been grown in a few cases. In 1945 Loo (152) grew excised stem tips of Asparagus officinalis on Bonner's basic medium (32) under light and by a series of subcultures was able to maintain them in culture for over nine months through 20 successive transfers. In darkness growth was decreased but at high sugar concentrations good growth was obtained even in the dark. Low sugar concentrations were needed for its growth in light.

In 1948 Galston (77) observed that freshly excised sterile asparagus stem tips formed roots readily when exposed to appropriate concentrations of IAA in the dark. No roots were formed in the light in the presence of the same concentration of IAA. If stem tips were cultured for several months in the dark they lost their ability to root in response to IAA although stem growth continued.

Smith (264) obtained a stimulation of the apical activity of shoot apices of wheat by 1 ppm gibberellic acid when the coleoptile was present with the shoot apex. His results suggested that gibberellic acid plays an important part in the regulation of growth in the apical meristem. The medium he used was that of Baldev (13) with 2 per cent sucrose. Petru (224) in studies with isolated shoot apices of wheat concluded that gibberellic acid stimulates the growth of cells in the subapical region and causes enlargement of the cells of the leaf primordia and that IAA does not alter this effect. At all concentrations which were studied kinetin alone or in combination with IAA produced inhibition and necrotization of the shoot apex if cultured without

leaves. These cultures were not grown on repeated subculture.

The studies on successful root tip cultures indicate the following factors which are of importance for continuous culture:

a) the choice of a suitable variety, since only some varieties of wheat and only a few of the rye cultures survived subculture,

b) supplementation of the basal medium (containing glucose or sucrose, salts and vitamins) with peptone or casein hydrolyzate, yeast extract and tryptophan in addition to auxin for some tissues,

c) a short subculture period.

SECTION 7

Callus cultures from endosperm, embryo and anther

(a) Endosperm culture

Maize

The first successful report of the culture of a cereal endosperm is that of La Rue (142). He grew maize endosperms on several media and obtained callusing of the exposed surfaces, which in some cases developed roots and in one case a root-shoot axis with miniature leaves. In 1949 La Rue (143) improved on his original medium and found that tomato juice could be used for culturing maize endosperm clone 1-c *in vitro*. However growth on media containing tomato juice was very erratic and unpredictable. In 1954 Straus and La Rue (295) studying further the nutritional requirements of Clone 1-c found that the erratic behaviour of the tissue could be corrected by substitution of tomato juice with 0.5 per cent filter sterilized yeast extract. The basal medium they used was White's (352) with Nitsch's (207) trace elements. An initial pH of 6.1 to 7.0 at 25° gave optimum growth.

Straus (288) made a comparative morphological and cytological study of normal maize endosperm when grown *in vitro* and found that cultured tissues were frequently multinucleate and polyploid, often possessing lobed nuclei of a bizarre appearance.

Sternheimer (273) reported a method for the culture of maize endosperm tissue excised 10 to 12 days after pollination. The medium used was White's (354) with 20 per cent clear tomato juice at pH 6.5.

Straus (289) also isolated endosperm callus from another maize variety, "Black Mexican Sweet". Two types of tissues grew - pigmented (red) and non-pigmented, the latter having a growth rate nearly double that of the pigmented tissue. A similar anthocyanin-containing strain of maize endosperm was also established by Sternheimer in 1954 (273). Straus (291) isolated the colorless and red tissues from maize endosperm and maintained them as separate cultures on a basal medium containing White's major elements and Nitsch's trace salts with 2 per cent sucrose. Asparagine was superior to yeast extract, casein hydrolyzate or tomato juice in supporting growth of the tissue, whereas coconut milk had no effect. These tissues also showed a strikingly high organic nitrogen requirement in the form of yeast extract or casein hydrolyzate. Straus (290) showed that maize endosperm cultures of "Black Mexican Sweet" had the capacity to produce three anthocyanin pigments. The tissue which earlier gave a deep purple pigment on subculture gave rise to a bright red one (which was quite different from the pigments isolated earlier. In 1960 (292) he found that the inhibiting agents of anthocyanin synthesis were riboflavin, methionine, asparagine, glutamine and valine, whereas the promoters of anthocyanin synthesis were aspartic acid and cystine.

Straus (293) found the presence of invertase in the cell walls of endosperm cultures of maize "Black Mexican Sweet". Later he and Campbell (294) observed peroxidase, amylase and acid phosphatase in the medium on which maize endosperm was grown. They (294) also found that cell wall IAA oxidase and peroxidase were enhanced when the cell wall preparations of the tissue were soaked in 0.05 M calcium chloride.

Tamaoki and Ullstrup (310) established tissue cultures of corn endosperm on a medium consisting of Knop's solution (131) with thiamine, pyridoxine, cysteine and Difco yeast extract. Sugary endosperm tissue

excised 8–11 days after pollination produced optimum growth. Moreover, established cultures on yeast extract medium did not respond to IAA or other substances such as kinetin or gibberellic acid.

Zea mays L. endosperm cultures were grown by Tulecke et al (330) on the synthetic medium used by Straus (292) in a phytostat. When this tissue was inoculated into the culture vessel, growth was very poor initially and after over a month only six grams of tissue per litre of medium were obtained. But the growth improved markedly after six weeks when a harvest of 15 to 20 grams per litre was obtained. Graebe and Novelli (94,95) used the colorless strain of maize endosperm "Black Mexican Sweet", for their studies on large scale plant tissue cultures and found that a single culture maintained in two 6-litre flasks yielded more than 5000 grams tissue over a period of 5 months. Using the same culture they obtained an active incorporation of leucine into protein from maize tissue culture cells when supplemented with the 105,000 x g supernatant fraction from maize seedlings.

Rye grass

Norstog (213) found that when the endosperm of English rye grass, Lolium perenne, was excised and placed on nutrient agar it produced a parenchymatous tissue capable of being subcultured indefinitely. The cultured endosperm grew most rapidly on White's medium supplemented with 0.25 to 0.5 per cent yeast extract increasing over 1500 per cent in weight in a 20 day culture period. Media containing coconut milk also gave good growth of the tissue. In 1969 cytological studies were carried out by Norstog et al (214) on these cultures which had been growing in culture since 1956 and several chromosomal aberrations were observed.

(b) Embryo callus culture

Carew and Schwarting (54) obtained callus tissue from rye embryos, variety 'Canadian Spring', on Heller's medium (103) supplemented with vitamins, 2 per cent sucrose, 0.5 per cent yeast extract and 1.0 ppm 2,4-D. Sym-diphenyl urea was also capable of supporting moderate growth when used in combination with casein hydrolyzate and 2,4-D. Neither adenine, kinetin nor gibberellic acid stimulated initiation of callus. These cultures were infected with Claviceps purpurea (55), but were unable to produce the clavine alkaloids. However in 1968 Chang and Carew (63) found that infection by Claviceps purpurea of rye callus growing on Murashige and Skoog's medium (193) modified by Lin and Staba (194) resulted in alkaloid production.

Maeda (154) succeeded in subculturing callus tissue derived from embryos of rice, and obtained organ formation. No information is available on its survival on subculture.

(c) Anther culture

Niizeki and Oono (205) obtained haploid callus from anthers of 6 varieties of rice on Blayde's medium (27) supplemented with IAA, kinetin and 2,4-D. This haploid callus when transferred to a medium containing IAA (2 ppm) and kinetin (2 to 4 ppm) formed organs and plants which had a haploid number of chromosomes. These cultures were not maintained on subculture.

Summarizing the results on continuous cultures of endosperms & embryos of cereals, the following factors appear to be of importance:

a) Basal salts with glucose or sucrose and an auxin.

b) A supplement such as yeast extract, casein hydrolyzate, tomato juice or asparagine was beneficial.

c) The choice of a suitable variety of seed.

SECTION 8

Callus cultures from root and stem tissues

(a) Non cereal

Amorphophallus: Morel and Wetmore (187) were the first to culture a tissue from a monocotyledonous plant. They obtained callus tissue from the tuber of Amorphophallus rivieri on Gautheret's medium (84) containing vitamins and NAA and were able to maintain its growth on subculture on a similar medium containing 15 per cent coconut milk from green coconuts. The tissues formed were compact and irregularly-surfaced colonies made up of homogeneous, large-celled parenchyma rich in starch, within which appeared a few clusters of irregular xylem elements and also scattered bundles of raphides.

Liliaceae and Amaryllideae: Robb (237) cultured explants from bulb scales of Lilium speciosum on White's basal medium (353) with vitamins and sucrose (2 per cent) and found that they proliferated and differentiated to regenerate bulblets within 15 to 16 weeks. Webster (343) described a method for the production of onion callus on Heller's medium modified by Carew and Schwarting (54) containing 2,4-D, EDTA and glucose, but this culture survived only for 12 months. Sheridan (257) successfully established callus cultures of Lilium longiflorum Thumb in agar and shake cultures on Linsmaier and Skoog's medium (150) containing major and minor salts, thiamine, inositol and 4 per cent sucrose. No other growth factors were necessary for growth or maintenance on subculture. In 1968 Klein and Edsal (129) and Krikorian and Katz (134) separately succeeded in growing onion callus cultures. Klein and Edsal used roots from seedlings of Allium cepa "Darjeeling Red", "Southport Red Globe",

and "Southport White Globe", and cultured them on a modified White's medium (130) supplemented with 6 ppm 2,4-D and 10 to 60 per cent CM. Callus formation had an absolute requirement for 2,4-D. Coconut milk below 20 per cent was inadequate and above 60 per cent inhibitory. Callus from these onion roots was also grown in liquid suspension culture. Krikorian and Katz (134) obtained callus formation from onion root tips (Trapp's strain of Downing Yellow Globe) under the influence of a CM medium with White's minerals (352) and 2,4-D or NAA. Mullin (189) obtained tissue cultures from 3 varieties of Allium cepa L on White's medium (355) with ferric EDTA, CM, 2,4-D (0.6 ppm) and sucrose (4 to 6 per cent). He also succeeded in culturing Spanish Bluebell (Endymion hispanicus) and a species of Hemerocallis L (daylily) on a similar medium containing CM and 2,4-D (6 ppm) and sucrose (4 per cent). A species of Crinum L could also be maintained on White's basal medium (355) containing CM, yeast and malt extracts, inositol and 2,4-D.

Orchideae: Morel (185) observed that when orchid meristems were placed on a basal medium containing CM and auxin, they proliferated with bud development in some cases. When this orchid meristem callus was passed through several media in sequence the free callus first formed clusters from which protocorms developed and eventually complete plants. Rao (231) obtained callus from seeds of orchids grown on Vacin and Went's (333) medium with 2 per cent sucrose and 1.6 per cent bacto agar, which later formed organized shoot apices. Steward, Mapes and Ammirato (287) obtained abundant plantlets from free cells of an orchid (Cymbidium species) grown initially on a basal medium containing CM and 2,4-D and then transferred to an NAA medium instead of 2,4-D and finally to a medium without any auxin. Mullin (189) cultured the beech orchid (Dendrobium falcorostrum Fitzg.) on the medium of Ranganathan et al (230) and maintained it through 15 passages.

Asparagus: Steward, Kent and Mapes (286) cultured a strain of Asparagus officinalis on a basal medium supplemented with CM and NAA for over 60 months and 20 subcultures. This culture however grew only slowly and its cells failed to show active protoplasmic streaming. When this tissue was transferred to a medium in which the NAA was replaced by 2,4-D, although at the outset the cell culture had not prospered under the influence of CM and 2,4-D it did so after prior growth on CM and NAA. In fact it then became an extremely vigorous culture containing cells and cell clusters which had all the features associated with a morphogenetically active culture from which whole plants were later obtained (275). In 1968 Wilmar and Hellendoorn (360) obtained callus of Asparagus officinalis L, derived from hypocotyls of sterile seedlings, on Linsmaier and Skoog's medium (150) with the addition of kinetin (0.315 ppm) and 2,4-D (1 ppm). In shake flasks embryoids appeared which attained geotropic orientation on solid media and which could later develop into plantlets on Difco orchid agar. Mullin (189) obtained callus cultures from Asparagus officinalis on White's basal medium (355) with ferric-EDTA and supplemented with CM, YE and 1 ppm NAA. The cultures survived through 15 passages and occasionally produced shoots.

Musa species

Banana: Mohan Ram and Steward (181) described a method for obtaining proliferative growth of explanted banana fruit tissue from a number of cultivated varieties of bananas at different stages of development. Explants weighing above 70 mg grew more rapidly than smaller pieces. White's culture medium (354) with 2 per cent sucrose and solidified with 0.5 per cent agar served as the basal nutrient medium. A number of supplements were tested for their effect on the growth of the explants and the best results were obtained with 2,4-D or 2-BTOA. Adenine in

combination with IAA enhanced the division of pulp cells. When the explants were grown in liquid media containing 2,4-D or 2,3,6-trichlorophenylacetic acid on a rotary shaker, a number of cells sloughed off into the bathing medium from which subcultures could be obtained.

(b) Gramineae (cereal cultures)

Sugarcane: In 1964 Nickell (197) established callus cultures from joints 4 to 10 of the internodal parenchyma tissue of 22 different varieties of sugarcane for the first time. White's basal medium (352) containing 18 per cent CM and 6 ppm 2,4-D was supplemented with 50 ppm ascorbic acid to prevent browning. Some of these cultures were also grown in liquid media. Maretzki and Nickell (157) showed the importance of arginine for tissue cultures of sugarcane grown in agar or in liquid medium containing YE and CM. Nickell and Maretzki (200) later cultured cell suspensions from sugarcane stalk parenchyma on a fully synthetic medium consisting of inorganic salts, vitamins, sucrose, 2,4-D and a mixture of 13 amino acids, the most important of which were arginine, aspartic acid and glutamic acid. Maretzki, Nickell and Thom (158) found that sugarcane cell cultures derived from parenchyma tissues of three different varieties showed a marked dependence on the presence of arginine. The cells took up exogenously supplied arginine rapidly and efficiently. A regulatory function for this amino acid was suggested since even a low level of this amino acid was effective in bringing about a growth response. Barba and Nickell (15) reported that Murashige and Skoog's medium (193) was inferior to White's (352) medium for supporting growth of callus cultures of sugarcane but was superior for fostering differentiation of roots or shoots or both from freshly isolated sugarcane cultures.

Nickell (198) studied the effect of different herbicides (delapon, diquat, parquat, endothal) incorporated at 20 ppm into White's medium containing coconut milk, arginine and 2,4-D on cultures of different sugarcane varieties. The cultures when exposed to delapon showed differentiation into organ-like structures.

Wheat: Trione et al (322) reported on their many unsuccessful attempts to culture somatic tissue of wheat on different media. Inter-cotyledonary node tissue grew well on Hildebrandt's medium (110) and produced masses of undifferentiated cells but did not survive on subculture. In 1968 Trione et al (323) grew somatic wheat tissue on Hildebrandt's 'D' medium (110) with NAA. When this tissue was transferred to Torrey and Reinert's (320) suspension culture medium with a very low auxin level the callus formed many roots. In 1964 Trione (321a) used the somatic callus cultures of "Elgin" and "Red Bobs" wheat for growing the bunt fungus Tilletia controversa.

Gamborg and Eveleigh (79) reported methods for the culture of 2 varieties of wheat in suspension culture. The cultures were derived from root sections of seedlings and cultured in a defined medium consisting of mineral salts, B-vitamins, sucrose and 2,4-D with nitrate and ammonia as sources of nitrogen. In the early periods of the cultures the cell aggregates readily produced roots, but this characteristic diminished after several generations of subculture.

Shimada et al (258) obtained callus from seedling roots and stem of three wheat varieties, Triticum aestivum, T.monococcum and T.dicoccum. The best callus growth was obtained on White's (352) or Risser and White's (236) medium containing 1 per cent CM, 0.1 per cent casein hydrolyzate and 0.2 to 2.0 ppm 2,4-D. Root formation took place in all media except those containing high 2,4-D concentrations (1 to 5 ppm).

Oat and barley

In 1966 Webster (343) described a method for the production of oat callus tissue by growing it on the basal medium of Carew and Schwarting (54) containing glucose and 2,4-D or IAA. The tissue survived for over three years. Carter et al (58) cultured oat tissue variety "Victory", on Linsmaier and Skoog's medium (150). Induction of callus and growth of the oat tissue were very sensitive to auxin (IAA or 2,4-D) concentration but were unaffected by kinetin.

In 1968 Gamborg and Eveleigh (79) reported methods for the culture of 2 varieties of barley on media similar to those used by them for culturing wheat.

Rye

Mullin (189) cultured rye tissues from a clone of excised roots of Secale cereale L, variety "Black Winter", on White's basal medium (355) with ferric-EDTA, 100 ppm inositol, 0.3 per cent, YE and 6 ppm 2,4-D. The tissue did not grow for more than 13 subcultures on this medium.

Sorghum

In 1970 Masteller and Holden (163) obtained growth of callus tissue of sorghum on Murashige and Skoog's medium (193) supplemented with CM and 2,4-D. When transferred to a medium with 5 ppm NAA instead of 2,4-D and kept under light, buds and roots appeared; these on transfer to pots with vermiculite and watered with Hoagland's solution (111) formed plantlets.

Pennisetum

Tiwari and Arya (315) obtained callus from healthy and diseased tissues of Pennisetum typhoides (bajra). The medium used was White's

basal (352) with acid hydrolyzed casein (Oxoid) and 2,4-D (9 ppm). This medium gave the best yield of tissue. A comparative study was also made on the growth requirements of callus derived from healthy and diseased tissue infected with Sclerospora graminicola in tissue culture. White's basal (352) mineral salts supplemented with acid hydrolyzed casein and 2,4-D proved best for both the tissues.

Rice

Yamada et al (363) obtained callus and subcultures from Oryza sativa variety 'Kyoto Asahi', on Linsmaier and Skoog's medium (150) without cytokinins and with only 2,4-D. Pieces of callus from the first subculture transferred to media without auxin and with different concentrations of kinetin formed shoots and roots (206).

Yatazawa (365,366) obtained callus from roots and nodes of young rice plants on a mineral salt medium with 2,4-D and yeast extract. The optimal temperature was 30° and glucose, sucrose or fructose could be used. The concentration of synthetic auxin required to develop the callus was 10-fold greater for IAA or NAA than for 2,4-D and the amount of auxin necessary to maintain growth of callus was less than that required for development of the callus. Allantoin as a nitrogen source increased growth of rice callus 2.5-fold (367,368).

These studies show no unusual nutritional requirements for monocot cultures. Generally the basal salts medium containing sucrose or glucose and vitamins required supplementation only with auxin (IAA, NAA or 2,4-D) for growth. The addition of CM, casein hydrolyzate, YE or malt extract was beneficial for many cultures. The requirement for arginine is unique for sugarcane cultures. No absolute requirement for a cytokinin or gibberellin could be demonstrated, but their presence in

some of the complex supplements such as CM or YE cannot be ruled out. No general conclusions appear to be possible regarding the influence of the variation of strain and the requirements for callus formation. However in many cases a high salt medium appears to be an important factor for growth of these cultures.

S E C T I O N 9

Summary of data on viable monocot tissue cultures

Table 1 summarizes data on monocot tissue cultures. Only those cultures which have been shown to be viable on continuous subculture have been listed. The cultures have been grouped into (1) Excised Root Cultures (2) Endosperm and Embryo Callus Cultures and (3) Root and Stem Callus Cultures. In each group the cereals (Gramineae) and non-cereals have been listed in separate subgroups. Data on the species, maintenance medium, year and bibliography reference number have been given for each culture and listed chronologically for each species.

TABLE 1

LIST OF MONOCOT CULTURES GROWN OVER MANY YEARS

(ORGAN AND CALLUS)

Species	Medium	Year	Reference No.
I. Excised Root Cultures			
Cereals			
1. *Secale cereale* Petkus II Winter Rye	Mineral salts with glucose + YE or tryptophan	1955	238
2. *Triticum vulgare* Hilgendorf 61	Mineral salts with vitamins + glucose + l-tryptophan	1963	70
Other monocots			
3. *Allium cepa* Trapp's strain of Downing yellow globe	Mineral salts with vitamins + CM + inositol + casein hydrolyzate + 2,4-D	1968	134
II. Endosperm and Embryo Callus			
Cereals			
1. *Zea mays* L endosperm clone 1-c	Mineral salts + tomato juice or YE + vitamins + sucrose	1949	143
2. *Zea mays* L. endosperm	Mineral salts with vitamins and sucrose + tomato juice	1954	273
3. *Zea mays* L Sugary endosperm	Mineral salts with vitamins + cysteine + Difco YE + sucrose	1958	310
4. *Zea mays* L Black Mexican Sweet endosperm	Mineral salts + Difco YE (or asparagine) +sucrose + vitamins	1960	292
5. *Lolium perenne* English Rye grass endosperm	Mineral salts + YE + sucrose with vitamins	1956	213
6. *Secale cereale* Canadian spring embryo	Mineral salts + vitamins + YE + 2,4-D + sucrose	1958	54

TABLE I (continued)

Species	Medium	Year	Reference No.
	III. Callus from Root & Stem Tissues		
Cereals			
1. *Saccharum officinarum* Sugarcane (many species) internodal regions	Mineral salts with vitamins + CM + 2,4-D + Vitamin C + sucrose	1964	197
2. *Zea mays* L Golden Bantam root or epicotyl	Mineral salts with vitamins + DPU + edamin + NAA + sucrose	1965	160
3. *Avena sativa* (Oat)	Mineral salts with vitamins + 2,4-D or IAA + glucose	1966	343
4. *Avena sativa* "Victory"	Mineral salts with vitamins + IAA or 2,4-D	1967	58
5. *Oryza sativa* 'Kyoto Asahi' Rice root	Mineral salts with vitamins + 2,4-D + sucrose	1967	363
6. *Oryza sativa* Rice (root and shoot)	Mineral salts with vitamins + YE + 2,4-D + sucrose	1967	366
7. *Oryza sativa* K 42 Rice (root and stem)	Mineral salts with vitamins + inositol + edamin + tyrosine + adenine + guanine + NAA + sucrose	1969	161
8. *Pennisetum typhoides* (Bajra)	Mineral salts with vitamins + Oxoid casein hydrolyzate + 2,4-D + sucrose	1967	315
9. *Triticum* (Wheat) Many varieties	Mineral salts with vitamins + NAA + sucrose	1968	323
10. *Triticum* (Wheat) 2 varieties	Mineral salts with vitamins + 2,4-D + sucrose + NZ-amine type A	1968	79
11. *Triticum vulgare* root and stem	Mineral salts with vitamins + inositol + edamin + NAA + DPU + sucrose	1969	161
12. *Triticum* (Wheat) 3 varieties	Mineral salts with vitamins + CM + casein hydrolyzate + 2,4-D	1969	258
13. *Hordeum* (Barley) 2 varieties	Mineral salts with vitamins + 2,4-D + sucrose + NZ-amine type A	1968	79
14. *Sorghum vulgare* Root and stem	Mineral salts with vitamins + inositol + edamin + NAA + DPU + sucrose	1969	161

TABLE I (continued)

	Species	Medium	Year	Reference No.
	Other monocots			
1.	*Amorphophallus rivieri* Stem	Mineral salts with vitamins + CM + NAA + sucrose	1951	187
2.	*Musa* species Banana fruit	Mineral salts with vitamins + casein hydrolyzate + an auxin	1964	181
3.	*Asparagus officinalis*	Mineral salts with vitamins + CM + 2,4-D	1967	286
4.	*Asparagus officinalis* hypocotyl	Mineral salts with vitamins + kinetin + 2,4-D	1968	360
5.	*Asparagus officinalis*	Mineral salts with vitamins + YE + NAA	1970	189
6.	*Allium cepa* Onion (3 varieties)	Mineral salts with vitamins + CM + 2,4-D	1968	129
7.	*Allium cepa* Trapp's strain of Downing Yellow Globe	Mineral salts with vitamins + 2,4-D or NAA + CM + inositol	1968	134
8.	*Allium cepa* 3 varieties	Mineral salts with vitamins + CM + 2,4-D + sucrose	1970	189
9.	*Lilium longiflorum* Thumb	Mineral salts with thiamine + inositol + sucrose	1968	257
10.	*Endymion hispanicus* Spanish Bluebell	Mineral salts with vitamins + CM + 2,4-D	1970	189
11.	*Hemerocallis* L Daylily	Mineral salts with vitamins + CM + 2,4-D	1970	189
12.	*Crinum* L	Mineral salts with vitamins + CM + YE + ME + inositol + 2,4-D	1970	189
13.	*Linope lour*	Mineral salts with vitamins + CM + 2,4-D	1970	189
14.	*Dendrobium falcorostrum* Beech Orchid	Mineral salts with vitamins + CM + YE + ME + 2,4-D	1970	189

SECTION 10

PRESENT INVESTIGATION

When this work was begun there were very few reports on viable callus cultures from stem and root regions of monocots and none from the cereals. A programme of work was, therefore, undertaken to obtain cultures from some of the economically important cereals, which would be viable indefinitely on subculture. Zea mays L "Golden Bantam" was first successfully established as a viable culture containing callus and roots, from sterile seedlings of this plant. A systematic study was then undertaken to determine the survival of this tissue on subculture. For this purpose the tissue was grown and subcultured repeatedly on a number of different media containing various supplements. These results indicated that maize callus tissue could be grown and maintained on either White's or Smith's mineral salt media containing an additional organic nitrogen source (in the form of casein hydrolyzate or edamin) and an auxin and glucose or sucrose as carbohydrate (160). Sym-diphenylurea appeared initially to be beneficial for survival of this tissue on subculture but further studies showed that the cultures could be maintained on repeated subculture without this compound. A higher auxin concentration (5 ppm) gave better results for callus formation and growth. Following these studies, callus tissues were obtained from root and epicotyl regions of wheat, rice and sorghum seedlings and subcultures were carried out over long periods. The stock media for wheat and sorghum were identical with the medium for maize, except that inositol was also added. Sorghum tissue also survived subculture on medium containing starch instead of sucrose. Rice tissue also grew well when this medium was supplemented with adenine, guanine and tyrosine. These results established that tissue cultures of maize, wheat, rice and sorghum were viable on subculture for several years. After confirming that these cultures were

viable in vitro, detailed studies were undertaken on their nutritional requirements with regard to mineral salts, organic and inorganic nitrogen, carbohydrates, auxins, kinins, gibberellins, vitamins and other compounds. In some cases the optimum concentrations of these nutrients were also determined (161).

When cultures of maize, wheat and sorghum were transferred from agar to liquid media and shaken on a rotary shaker, they grew as a mass of roots. The growth rate was also markedly higher in shake flasks than on agar. The optimum concentrations of NAA, vitamins, inositol and casein hydrolyzate and the effect of cytokinins and gibberellin as well as plant growth inhibitors on maize in shake flasks were also studied. The growth curves and rates of utilization of carbohydrate, organic nitrogen, phosphorus and iron were determined for maize and wheat in shake flasks.

Preliminary studies on differentiation of these cultures were carried out. The effect of gibberellic acid (GA_3) and of different auxin-kinin ratios on maize and wheat was investigated. Under no conditions was stem initiation observed, though root formation could be induced at low auxin levels or with GA_3 in static agar cultures; whereas even at high auxin levels only root formation was observed in shake flasks.

Biochemical changes taking place in maize and wheat cultures during the transition of callus to roots in agar and liquid media were studied by assaying a few enzymes of the glycolysis and hexose monophosphate pathways and of the citric acid cycle as well as a few hydrolytic enzymes. No major differences between callus and root cultures were observed in their enzyme content.

Chapter I of this thesis deals with the materials and experimental methods used in these studies.

Chapter II deals with the initiation of callus obtained from seedling explants of maize, wheat, rice and sorghum and their survival over long periods of subculture.

Chapter III deals with nutritional studies of maize, wheat, rice and sorghum on agar media.

Chapter IV deals with the growth of maize and wheat in liquid medium in shake flasks, and the utilization of some of the nutrients from the medium.

Chapter V deals with studies on differentiation of these cultures.

Chapter VI deals with the chemical and enzyme composition of maize and wheat tissues grown on agar and liquid media.

Chapter VII gives a summary of the results of these experiments and the main conclusions from these studies. A bibliography concludes this thesis.

CHAPTER I

MATERIALS AND METHODS

Pages 39 - 53

C O N T E N T S

SECTION 1

Materials

The inorganic salts used for the preparation of media, and the carbohydrates were of analytical grade (British Drug Houses or E. Merck). The substrates used for the different enzyme assays, auxins, kinins and gibberellins and the other chemicals were pure preparations from the sources indicated in parenthesis: Yeast and malt extracts, bacto-casamino acids (casein hydrolyzate) and bacto-agar (Difco), auxins, vitamins, nucleotides, kinins, amino acids (Sigma Chemical Company or British Drug Houses), AMO-1618, enzyme substrates (California Biochemical or Sigma Chemical Co.), corn steep liquor (Anil Starch Products, Ahmedabad).

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; Edamin from Sheffield Chemical Company, New York; abscisic acid (II) from Hoffmann La Roche, Basle; gibberellins (GA_1, GA_3, GA_4, GA_5, GA_{4+7}, GA_7, GA_9, GA_{13}) from Imperial Chemical Industries, U.K.

The seeds used in these studies were obtained from the following sources:

Zea mays L variety "Golden Bantam' (maize)- P. P. Pocha & Sons, Poona; Oryza sativa L variety K_{41} (rice) Agricultural College, Poona; Triticum vulgare L commercial variety (wheat) - local market; Sorghum vulgare L yellow seeds commercial variety (sorghum)-

local market.

Ferric ethylehe diamine tetraacetic acid was prepared according to Murashige and Skoog (193). Coconut milk was obtained by pooling together the filtered water from a number of tender green coconuts, autoclaving at 15 psi for 20 minutes and storing at -20°C. Before use it was thawed and filtered. Dowex-50 (200-400 mesh, 8 per cent cross linked) was converted to the hydrogen form and Dowex-1 (200-400 mesh 8 per cent cross linked) to the acetate form before use.

Glassware

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

SECTION 2

Estimations

a) Nitrogen, phosphorus, iron and sugars

Nitrogen was estimated in the medium and the tissue by the micro-Kjeldahl method and total phosphorus by the procedure of Fiske and Subba Row (73). For the estimation of sugar in the medium, an aliquot of the medium was hydrolyzed with 1N hydrochloric acid in a water bath at 100°C for 15 minutes, cooled and then neutralized with 1N sodium hydroxide. The sugars were estimated according to Bertrand and Thomas (24) and the results expressed as sucrose. Iron was estimated colorimetrically according to Sandell (245) using o-phenanthroline.

b) Sugars

Tissues of maize and wheat were weighed and extracted 3 times with hot 70 per cent ethanol (281). The 70 per cent ethanol extract was passed through 1 x 5 cm columns of Dowex-50 (H^+ form) at a flow rate of 3 ml per minute to remove amino acids (226). The eluate obtained was passed through 1 x 10 cm columns of Dowex-1 acetate to remove organic acids (345). The eluate which contained the sugars was taken to dryness under vacuum. The dry residue was dissolved in a small volume of water, shaken with chloroform to remove pigments and the aqueous layer kept for sugar analysis. The identification of the sugars was carried out by one dimensional descending paper chromatography on Whatman number 4 filter paper. Runs were carried out using two different solvent systems: butanol:acetic acid:water (4:1:5), and phenol saturated with water (223a). Guide strips of known sugars and of the tissue extracts were developed with 0.2 per cent naphthoresorcinol in ethanol and orthophosphoric acid (9:1, v/v) and dried at 90° for 5 minutes (41). Corresponding areas

were then cut from undeveloped portions of the chromatograms and these were eluted with hot water and the eluate filtered through glass wool. The eluate was dried under a stream of warm air. Two ml of water were added followed by 4 ml of a 2 per cent solution of anthrone in concentrated sulphuric acid, the tubes were allowed to stand for 10 minutes and the optical density was read at 640 mu in a Beckman DU spectrophotometer (280).

c) RNA and DNA

The method used for the extraction of DNA and RNA from the tissues was modified from Fleck and Munro (74) as follows:

The tissue (500 mg) was ground in a chilled mortar with 10 ml of 0.2 N perchloric acid for the removal of acid soluble material and kept in perchloric acid for 30-40 minutes at 0°. The extract was centrifuged at 0-4°C and the supernatants discarded. To the residue 4 ml of 0.3 N KOH were added. It was then incubated at 37-40° for 2 for 3 hours for dissolution of RNA. It was cooled to 0° and perchloric acid added to make the final concentration 0.2N with respect to perchloric acid. It was centrifuged at 0-4° and the precipitate of DNA was dissolved in hot 0.5N perchloric acid (2 ml) at 70-80° for 45 minutes and then centrifuged. The supernatant liquid contained DNA which was estimated by the diphenylamine reaction according to Burton (47). RNA from the perchloric acid extract was determined by measuring the extinction coefficients at 260 mu and correcting for protein according to the method of Barker and Hollinshead (16). Protein was determined by Lowry's method (153).

d) Protein

Protein was measured spectrophotometrically in a cuvette of 1 cm light path by a modification of the method of Warburg and Christian (342) using the following formula for correcting for nucleic acid (119):

$$\text{mg protein per ml} = \frac{4}{7}\left[2.3\ (\text{O.D. 280 mu} - \text{O.D. 340 mu}) - (\text{O.D. 260 mu} - \text{O.D. 340 mu})\right] \times \text{dilution}$$

It was assumed that 0.1 per cent protein solution has an optical density of 1 at 280 mμ.

e) Enzyme assays

The enzymes in the tissue extract were assayed by the methods given in the literature

Lactate dehydrogenase (136); glucose 6-phosphate dehydrogenase (341); 6-phospho gluconate dehydrogenase (114); hexokinase (115, 124); isocitrate dehydrogenase (91); alcohol dehydrogenase (228); malate dehydrogenase (113); pyruvate kinase (1); phosphoenolpyruvate carboxylase (332); deoxy ribonuclease (137); ribonuclease (244); β-glycerophosphatase (252); α-amylase (23); esterases (116).

f) Cell counts

Cell counts were made from samples of fresh tissue, each weighing over 50 mg and harvested at different growth intervals. Each sample was immersed separately in 5 ml of a 1:1 solution containing 5 per cent (w/v) chromic acid in water and 0.5 N hydrochloric acid. After 12 hours at 26° the cell masses were broken by gentle in and out movements using a teflon coated Potter Elvehjem homogenizer. The cells were counted on a haemocytometer slide. Six haemocytometer readings from each tissue sample were made and the average number of cells was calculated (9,89).

SECTION 3

Composition of media

The composition of the different basal media used in the course of this work is shown in Tables 2A, 2B and 2C. The supplements added to the basal media and their concentrations are described in the text. White's and Knop's half strength media were prepared according to White (354). Hildebrandt's medium was the 'D' medium for tobacco tissue (110). Murashige's medium was made according to Murashige and Skoog (193) and Smith's medium (265) according to a personal communication from Dr. W. C. Smith, U. K. However, all the media were modified as described below. "White's", "Smith's" medium etc. therefore refer not to the media described in the original publications,but to the modified media the compositions of which are given below. The macro elements (Table 2A) in all the media were added exactly as described in the original publications. Among the micro elements (Table 2B) iron was added to all the media as ferric EDTA at the concentrations shown in the Table, instead of ferric sulphate (White's) or ferric tartrate (Smith's). The trace elements added to Knop's, Murashige and Skoog's& Hildebrandt's media were according to Smith (Table 2B) and not as in their original compositions. All media, therefore, had the trace element compositions shown in Table 2B according to "White" or "Smith". Copper and molybdenum were added to White's 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. (In cases where iodide was omitted from the medium it has been specified in the Table). Changes in the micro-element and vitamin composition from the original media are marked with an asterisk (*) in Tables 2B,and 2C. Thiamine, pyridoxine,

TABLE 2A

INORGANIC SALTS USED IN PREPARATION OF DIFFERENT MEDIA

MACRO ELEMENTS

(Concentrations expressed as mg/l of medium)

Chemical	White	Smith	Murashige & Skoog	Knop	Hildebrandt
$Ca(NO_3)_2 \cdot 4H_2O$	288	710	-	144	400
NH_4NO_3	-	-	1650	-	-
KNO_3	-	405	1900	25	80
$CaCl_2 \cdot 2H_2O$	-	-	440	-	-
Na_2SO_4	200	-	-	-	800
KCl	80	-	-	-	65
$NaH_2PO_4 \cdot 2H_2O$	22	-	-	-	47
KH_2PO_4	-	70	170	25	-
$MgSO_4 \cdot 7H_2O$	720	245	370	51	180
NaCl	-	60	-	-	-

TABLE 2B

INORGANIC SALTS USED IN PREPARATION OF DIFFERENT MEDIA

MICRO ELEMENTS

(Concentrations expressed as mg/l of medium)

Chemical	"White"	"Smith"
Na_2-EDTA	37.30*	37.30*
$FeSO_4.7H_2O$	27.80*	27.80*
$ZnSO_4.7H_2O$	2.60	-
$ZnCl_2$	-	0.62
$MnSO_4.H_2O$	5.00	-
$MnCl_2$	-	0.49
H_3BO_3	1.50	0.57
KI	0.75	0.75*
$CuCl_2$	0.27*	0.27
$Na_2MoO_4.2H_2O$	0.25*	0.25

*indicates difference from the original media

TABLE 2C

VITAMINS AND GLYCINE ADDED IN PREPARATION OF MEDIA

(concentrations expressed as mg/l)

	Chemical	
1	Thiamine HCl	1*
2	Pyridoxine HCl	1*
3	Nicotinic acid	5*
4	Glycine	30*

*indicates difference from White's vitamin solution (354).

TABLE 2D

THE COMPOSITION OF DIFFERENT CULTURE MEDIA USED IN THESE STUDIES

(The abbreviations used for the respective media are given in Column 1)

Concentration of inorganic salts, vitamins and glycine as in respective Tables. Sucrose was added at 2% to all media.

Medium	COMPOSITION					
	Macro salts	Micro salts	Vitamins & Glycine	Organic Nitrogen	Other Supplements	NAA
				ppm	ppm	ppm
WB	Table 2A (White)	Table 2B (White)	1/10 concentrations shown in Table 2C	-	-	-
WB_1	"	"	"	-	-	1
WB_2	"	"	Table 2C	-	-	1
WB_3	"	"	Table 2C	-	-	5
BM	Table 2A (Smith)	Table 2B (Smith)	1/10 concentrations shown in Table 2C	Edamin (1000)	-	1
BM_1	"	"	Table 2C	Edamin (1000)	Inositol (100)	1
BM_2	"	"	Table 2C	Edamin (1000)	Inositol (100) DPU (1)	5
BM_3	"	"	Table 2C	Edamin (1000)	Inositol (100) DPU (1) Adenine (1) Guanine (1) l-Tyrosine (2)	5
BM_2P	Table 2A (Smith) with KH_2PO_4 140 mg/ litre instead of 70 mg	Table 2B (Smith)	Table 2C	Edamin (1000)	Inositol (100) DPU (1)	5

nicotinic acid and glycine were added to most media at the concentrations shown in Table 2C. These concentrations are ten times higher than those used by White (354). In most of the initial experiments given in Chapter II on the initiation of callus from maize, these vitamins and glycine were, however, added at the concentrations given by White.

The sucrose concentration was 20 g/litre and that of agar 8 g/litre, unless otherwise indicated. Any changes in the above media are shown in the corresponding tables or in the text.

Abbreviations have been given in Table 2D to describe the compositions 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 semi-solid media agar was added at 0.8 per cent. The medium was steamed for 30 minutes to melt the agar, and transferred in 20 ml lots to test tubes (25 x 150 mm). In shake flask cultures 50 ml liquid medium (without agar) was transferred to 250 ml Erlenmeyer flasks. Sterilization was carried out by autoclaving at 15 psi for 20 minutes, followed by steaming for 30 minutes on the subsequent day. Heat sensitive compounds were sterilized by passing through a Seitz bacterial filter and 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 4

Methods of subculture and growth measurement

a) Inoculations

All inoculations were carried out in a sterile room under an inoculation hood provided with an ultraviolet lamp which was switched off during inoculations. A continuous stream of air, which was sterilized by passing through aluminium strips coated with glycerine followed by irradiation with UV light, was passed through the room. Sterile forceps were used for transferring the plant tissues during the inoculations and subcultures. For growth experiments on agar, callus tissue pieces of approximately uniform initial weight, as judged visually, were removed aseptically from a six week old agar culture and inoculated into the test media, one piece per tube. Maize, wheat and sorghum tissue used as inoculum for growth experiments was grown on BM_2 medium, and rice on BM_3. The wet weight of maize, wheat and rice inoculum was between 80 to 90 mg per tube whereas the inoculum used for sorghum was 30 to 40 mg.

The growth measurements were generally made at the end of 40 to 45 days after which time the wet and dry weights were determined. The average growth of five replicate cultures was measured by Steward (274) in his growth studies. A similar number of cultures was kept in the present investigation. Moreover the number of tubes required for these experiments was so large (about 20,000 in all the experiments) that it was not feasible to use a greater number of replicates.

For growth experiments in shake flasks 200 to 300 mg of fresh tissue from 18 day old shake cultures were transferred to test media.

Shake flask experiments were carried out in duplicate and the final growth measurements, given as the wet and dry weights are the average of two flasks at the end of 15–20 days incubation. The tissue grown in shake flasks was gently pressed between folds of filter paper to remove traces of medium before weighing. The variation in the fresh weight between the 2 flasks was generally about ±200 mg for a growth of 2 to 4 g of tissue.

b) Incubation of cultures

After inoculation the cultures were incubated in a room at 25°C±1°C and illuminated with diffuse artificial light from fluorescent tubes (5 foot candles) for about 4 hours per day. The relative humidity of the room was maintained at 60 to 70 per cent. Liquid cultures were transferred to a rotary shaker with a 5 cm amplitude and shaken at 125 to 180 revolutions per minute.

Stock cultures of maize, wheat, rice and sorghum grown on agar were maintained on different media (Chapter II), subcultures being made into the respective media every 40 days. Stock cultures of maize in shake flasks grew as roots and were subcultured every 18 days (Chapter III) into different media.

c) Measurement of growth

Wet and dry weights were taken as a measure of the growth of the tissues in agar and liquid media. Dry weights were obtained by drying the tissue to constant weight at 95 to 100°C. In comparing growths dry weight was taken into account to a greater extent than fresh weight. Changes in fresh and dry weights in general ran parallel, but in some cases the water content varied considerably.

d) Standard error

To determine the difference in weight between replicate cultures, an experiment was set up in which maize, wheat, rice and sorghum tissues of uniform inoculum size were transferred into 10 tubes each of their respective media (BM_2 for maize, wheat and sorghum and BM_3 for rice). After 40 days the weight of the tissue in each of the 10 tubes was taken and the standard error obtained by the formula $\pm\sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}$ where Δ is the difference between the individual value and the average and n the total number of tubes (193). The size of inoculum and the final fresh weights are given in Table 3 in mg for each cereal together with standard errors of the mean.

TABLE 3

STANDARD ERROR IN INITIAL AND FINAL FRESH WEIGHT OF MAIZE, WHEAT, RICE AND SORGHUM

	Inoculum weight	Final weight
Maize	85±5	340±56
Wheat	86±4	430±72
Rice	90±3	670±54
Sorghum	40±2	152± 9

These figures indicate that the standard error of the final weight, obtained for maize and wheat was about 16-17 per cent, for rice ±8 per cent and for sorghum ± 6 per cent. The differences in inoculum size were less than 6 per cent. The results of experiments in which there was a visibly different growth in replicate tubes (i.e., markedly higher and lower growth rate or absence of growth in one or more tubes) were discarded.

It should be noted that the growth rate of several of these cultures increased markedly with subculture, for example after 2 to 3 years the growth rate was greater than in the first year (see Chapter II, Section 1). Hence the growth rate even under identical conditions was not the same in experiments carried out at different periods. Since the experiments described in this work were carried out over a period of five years there is considerable variation in growth rates. The data in each table are comparable but the data in two different tables which might have been carried out at different periods are not necessarily comparable. It should also be noted that the results are not always presented in the chronological order in which they were carried out.

CHAPTER II

INITIATION OF CALLUS AND STUDIES ON SURVIVAL OF MAIZE, WHEAT, RICE AND SORGHUM ON SUBCULTURE IN VITRO

(Pages 54 - 77)

C O N T E N T S

Initiation of callus and studies on survival of maize, wheat, rice and sorghum cultures in vitro

Callus tissues from dicotyledonous plants are easily obtained on media containing mineral salts, a carbohydrate source and often an auxin and/or a kinin. However, very few monocotyledonous tissues have been grown in vitro. The inadequacy of the media used for isolation of callus cultures is one possible reason for the failure of earlier attempts at culture of these tissues. Several different media were therefore tested for this purpose, both for the initiation of callus and for subculture of these tissues. The possibility that media suitable for the initiation of callus and those required for continued growth on subculture could be different was also taken into account. The main objectives of this work were to obtain viable cultures of the cereals which could be maintained indefinitely by subculture in vitro and to find chemically defined media for their growth so that their nutritional requirements could be ascertained in terms of known constituents.

This chapter describes the conditions for the initiation of callus from segments of seedlings of maize, wheat, rice and sorghum and a study of their survival on subculture. Greater emphasis has been given to maize tissue since this was the first cereal to be studied; from the knowledge gained with this tissue the other cereals were then grown on media containing the different supplements tested for maize.

S E C T I O N 1

a) Initiation of callus from maize

About 40 to 50 seeds of the cereal were first surface sterilized as follows. (All operations were carried out under aseptic conditions and all solutions, instruments and glassware were previously sterilized.) The seeds were thoroughly washed with distilled water, immersed in 70 per cent alcohol for 5 minutes and then washed with distilled water. They were then shaken for five minutes with 50 ml of distilled water containing a few flakes of soap or detergent. The liquid was poured off and the seeds were washed once again with distilled water. They were then transferred to a flask containing 100 ml of a 3 per cent sodium hypochlorite solution and shaken for 15 minutes. The hypochlorite solution was discarded and the seeds were washed with sterile distilled water till no trace of hypochlorite could be detected.

The seeds were then transferred to test tubes containing cotton moistened with distilled water and kept for germination at 25° in the dark. They generally germinated on the fourth or fifth day. After germination 4 to 5 mm segments from the root, epicotyl or stem regions of the seedlings were transferred aseptically into test tubes containing several different media (Table 4) and the growth of the segments and initiation of callus on different media were observed. Callus formation was often accompanied by a simultaneous growth of the root or stem segment cultured. Plate 1 shows callus forming from seedling explants of maize.

PLATE 1: CALLUS FORMATION FROM MAIZE SEEDLING EXPLANTS

(1) EPICOTYL (2) ROOT (3) STEM

TABLE 4

INITIATION OF CALLUS FROM SEEDLING SEGMENTS OF MAIZE ON DIFFERENT MEDIA

Mineral salts: (Macro and micro) Modified White's or Smith's (Tables 2A, B), KI was omitted from Smith's micro salts (Table 2B) (Media 11,12,13,14).
Sucrose at 2%.

Concentration of supplements: (%) CM (15); Corn steep liquor (CSL) (0.2); (ppm) - DPU (1); edamin (1000); inositol (100).

Inoculum: 5 mm seedling segments (root or stem).

Growth measurements after 45 days (mg): +, 30–40; 2+, 40–50; 3+, 60–80; 4+, over 100; -, tissue dies.

1 to 10: Series I; 11 to 14; Series II.

Series II are the additional media used at the second attempt to initiate callus.

	Mineral salts	Vitamins + glycine Table 2C	Other supplements	Auxin	Auxin concentration	Callus growth
1	White	1/10 concentration in Table 2C	-		ppm	-
2	White	"	-	NAA	1	+
3	White	"	CSL	NAA	1	++
4	White	"	CM	NAA	1	+
5	White	"	DPU	NAA	1	++
6	White	"	DPU, CSL	NAA	1	+++
7	White	"	CM, CSL	NAA	1	+++
8	White	"	DPU, CSL	2-BTOA	5	-
9	White	"	DPU, CSL	2,4-D	0.6	-
10	White	"	Edamin	NAA	1	+++
11	Smith	"	DPU, edamin	NAA	1	+++
12	Smith	"	DPU, edamin,inositol	NAA	1	+++
13	Smith	Concentration as in Table 2C	Edamin, inositol	NAA	5	++++
14	Smith	"	DPU, edamin, inositol	NAA	5	++++

The effect of different media on the growth of tissue is shown in Table 4. White's medium was used in the first series of experiments. Coconut milk (CM), diphenyl urea (DPU), corn steep liquor (CSL), edamin and different auxins were the supplements tested. Corn steep liquor was tested since it is derived from maize and may contain growth factors for maize tissue in addition to amino acids, vitamins etc. Stimulation of growth was observed in the presence of this supplement. With CM slight growth stimulation was observed. Since CM was shown to contain DPU by Shantz and Steward (255) this compound was also tested individually on the growth of maize explants and found to cause growth stimulation. A combination of corn steep liquor and DPU with or without CM gave the maximum growth of this tissue. It is obvious from the Table that NAA is essential for growth and that the other auxins (BTOA and 2,4-D) were ineffective. Edamin, an enzymatic hydrolyzate of lactalbumin, was also tested and found to stimulate growth markedly.

Since White's medium has a low salt concentration the effect of Smith's mineral salt medium, which contains a higher concentration of some inorganic nutrients, was tested in the second series of experiments. In experiments 11 and 12, the concentration of vitamins and glycine was that used by White, whereas in experiments 13 and 14 these substances were added at a ten times higher level. Edamin was added in all four media of the second series. The effect of inositol, DPU and different concentrations of NAA was also tested. Growth of callus was good in all the experiments of this series, but on the high vitamin - high NAA media maximum growth was observed.

b) Growth of maize tissue on subculture

The effect of different media on survival of maize cultures was then studied. In the case of experiments in which the cultures did not survive, only the media combinations are shown (Table 5). The growth was often good with some media in the initial subcultures but decreased after 5 to 6 subcultures and the tissue failed to survive. None of the supplements (edamin, coconut milk, kinetin, zeatin, corn steep liquor or DPU singly or in different combinations) gave tissue cultures which were viable on subculture.

In three media (Figure 1) maize tissue was found to grow well on subculture. These media contained DPU or corn steep liquor along with DPU or coconut milk. These cultures were carried through 14 subcultures but unfortunately all the cultures suddenly died, possibly due to an impurity in a sucrose sample used for this work. It should be noted that all the media shown in Table 5 and Fig. 1 contained vitamins and glycine at one-tenth the level shown in Table 2C and that the basal medium was based on White's medium.

A new set of experiments was then initiated and 18 different media were tested for their effect on the growth and survival of maize tissue. It will be seen from Table 6 that the cultures were viable on all the media for 32 to 56 months (at the time of writing this thesis). (Some of the experiments were started much later than the others on the basis of the results obtained in subsequent experiments with maize or other cereal cultures.) It was not possible to study all the combinations

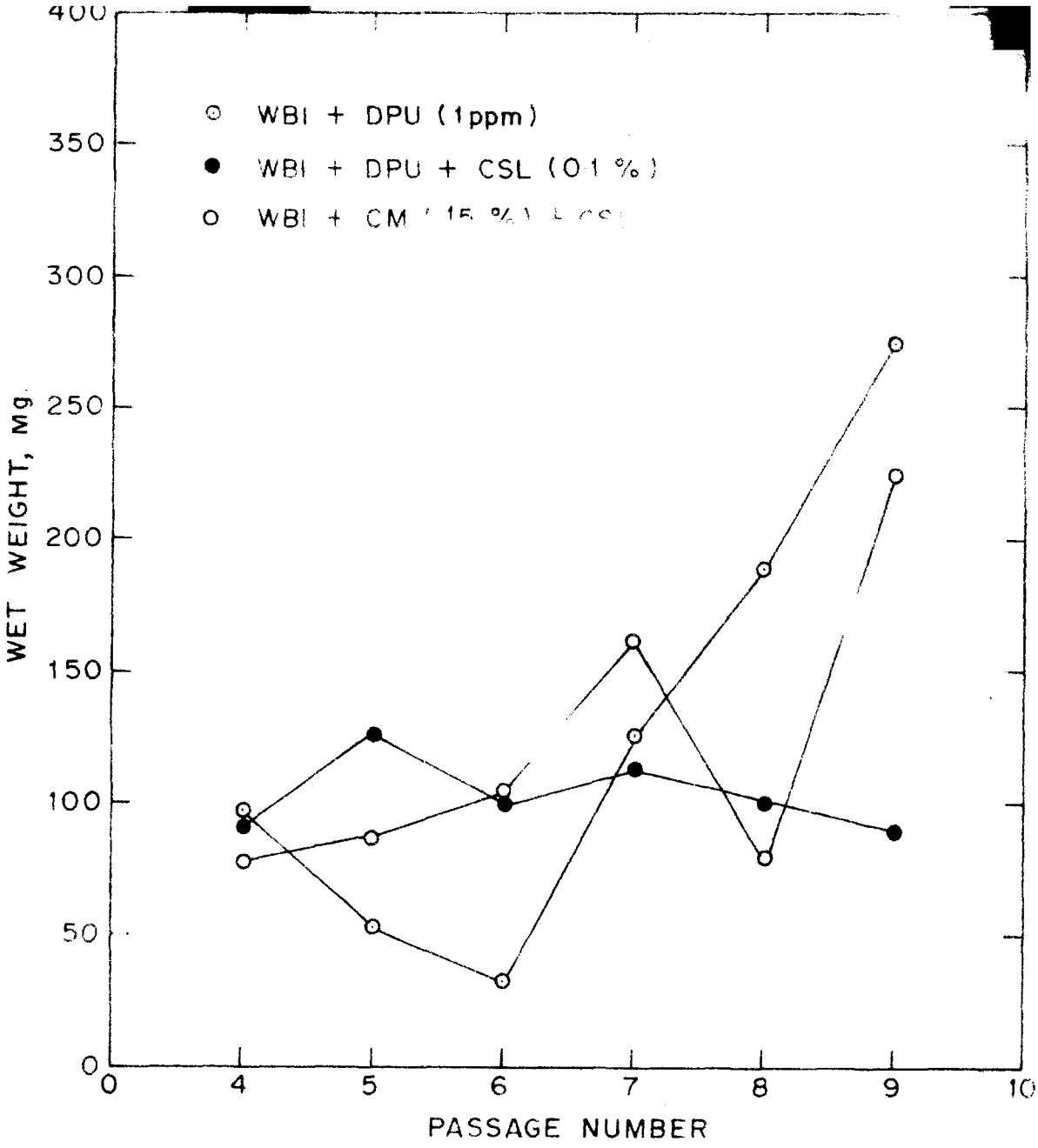


FIG. 1.

EFFECT OF SUBCULTURE ON THE GROWTH OF MAIZE TISSUE IN DIFFERENT MEDIA.

TABLE 5

SOME MEDIA ON WHICH MAIZE TISSUES DID NOT SURVIVE ON SUBCULTURE

Basal medium: Mineral salts (macro and micro) White's - Tables 2A, 2B
Vitamins and glycine: 1/10 concentration given in Table 2C,
Auxin: NAA (1 ppm).

Supplements added to basal medium: Concentration % or ppm : CM (15), CSL (0.2), DPU (1), kinetin (1), edamin (1000); zeatin (0.1 µg/l).

Inoculum: 10-15 mg

Period (days): 45

	Supplements
1	-
2	Edamin
3	Edamin + DPU
4	Edamin + CSL
5	Edamin + CM
6	Edamin + CM + CSL
7	Edamin + CM + DPU
8	Edamin + DPU + CSL
9	Edamin + DPU + CM + CSL
10	CM
11	Kinetin
12	Zeatin
13	Kinetin + DPU
14	Kinetin + CSL
15	Kinetin + DPU + CSL
16	CSL

of nutrients required to establish the essentiality of each of the components. Some important conclusions can however be drawn from the results of Table 6. For continuous growth of maize cultures either White's or Smith's mineral salts can be used with sucrose, glucose or starch as carbohydrate. With a higher vitamin concentration the growth obtained was superior (media 5 and 6). NAA was essential, the growth with 5 ppm being better than with 1 ppm (medium 18).(All cultures failed to survive in an auxin-free medium.) Inositol, DPU, KI, adenine, guanine were not essential for the survival of the culture. The tissue also survived repeated subculture on a medium containing NH_4NO_3 in addition to nitrate present in Smith's medium. On this medium root formation was greatly increased. Edamin could be replaced by an acid hydrolyzate of casein. In earlier studies (Table 5), media containing edamin failed to support survival of maize tissue on subculture, but these media contained lower levels of vitamins and glycine. It is not known whether this was the reason for the non-viability of the cultures on several of the media shown in Table 5.

The maize tissue grown on the different media which ranged from simple media supplemented with a protein hydrolyzate and auxin to more complex media containing many supplements need not necessarily be identical. It is possible that the initial callus from the explants was heterogeneous and during long term culture on different media different types of cells may have undergone selection. Hence the cells present in one medium may be different from those on another. A comparison of the growth rates on two different media may not necessarily be meaningful since the same type of cells may not be present on the two media. It has

TABLE 6

EFFECT OF DIFFERENT MEDIA ON GROWTH AND SUBCULTURE OF MAIZE TISSUE

Mineral salts: Modified White's or Smith's (Tables 2A & 2B). KI omitted from some media given in Table. Vitamins and glycine added according to Table 2C, except in medium 5* at 1/10 Table 2C concentration.

Supplements: (ppm) - edamin (1000); casein hydrolyzate (CH) (1000); inositol (100); DPU (1); adenine (1); guanine (1); tyrosine (2); NH_4NO_3 (100); NAA (1 or 5); carbohydrate (2%).

Inoculum: 50-60 mg

Period (days): 45

	Mineral salts	KI	Carbo-hydrate	Edamin or casein hydroly-zate	DPU	Other supplements	NAA	Wet wt. at last passage	No. of passages	Period of growth
							(ppm)	(mg)		(months)
1.	White's	+	Glucose	Edamin	-	-	1	167	37	56
2.	"	+	Sucrose	CH	-	-	1	186	37	56
3.	"	+	Glucose	Edamin	+	Inositol	1	219	37	56
4.	"	+	Sucrose	CH	+	Inositol	5	231	37	56
5.	*Smith's	-	Sucrose	Edamin	-	-	1	208	21	32
6.	"	-	Sucrose	Edamin	-	-	1	404	38	57
7.	"	-	Sucrose	Edamin	+	-	1	233	33	49
8.	"	+	Sucrose	Edamin	-	Inositol	1	220	33	49
9.	"	+	Sucrose	Edamin	-	Inositol, adenine, guanine	1	390	32	47
10.	"	+	Sucrose	Edamin	-	Inositol, adenine, guanine, tyrosine	1	344	26	38
11.	"	+	Sucrose	Edamin	-	Inositol, NH_4NO_3	1	180	29	45

TABLE 6 (contd.)

	Mineral salts	KI	Carbo-hydrate	Edamin or casein hydroly-zate	DPU	Other supplements	NAA	Wet wt. at last passage	No. of passages	Period of growth
							(ppm)	(mg)		(months)
12.	Smith's	+	Glucose	Edamin	-	Inositol	1	180	33	49
13.	"	+	Glucose	Edamin	+	Inositol	1	175	33	49
14.	"	-	Starch	Edamin	-	Inositol	1	380	32	47
15.	"	+	Starch	Edamin	-	Inositol	1	294	32	47
16.	"	-	Starch	Edamin	+	Inositol	1	380	32	47
17.	"	+	Starch	Edamin	+	Inositol	1	300	32	47
18.	"	+	Sucrose	Edamin	+	Inositol	5	340	33	49

unfortunately not so far been possible to obtain clones derived from single maize cells, though attempts to define the conditions for the growth of single cells of the cereals are in progress.

As a result of these experiments the basic medium selected for further studies on the nutritional requirements of maize (Chapter III) was BM_2 i.e., Smith's basal containing higher levels of vitamins, glycine and NAA and supplemented with inositol, edamin and DPU unless otherwise specified.

SECTION 2

Initiation of callus and studies on survival of wheat, rice and sorghum on subculture

a) Initiation of callus

Sterile seedlings of wheat, rice and sorghum were grown as in the case of maize and 5 mm segments dissected and inoculated into White's or Smith's basal media containing different supplements that were effective in growing maize tissues. In addition adenine, guanine and cytidylic acid were added to the media for rice since the growth of this tissue on the media found suitable for wheat or sorghum was relatively slow.

Wheat: Callus induction was observed on both root and stem segments on Smith's media containing NAA with either CM, DPU or edamin and with combinations of CM or edamin with DPU (Media 4,5, 6,7 and 8, Table 7). NAA at 5 ppm added to a Smith's medium supplemented with DPU, edamin and inositol gave maximum callus formation (Medium 9).

Rice: For initiation of callus from rice seedling segments, additional supplementation of adenine, guanine and cytidylic acid or tyrosine to Smith's, edamin, inositol, NAA medium was found to give better growth (Media 11, 12 and 13, Table 7). NAA was added only at 5 ppm. Callus growth was observed with or without DPU (Media 9 and 10).

Sorghum: The minimum requirements for callus formation from sorghum seedling explants was White's medium with NAA and either CM or DPU (Media 1,2,3, Table 7). Optimum growth was observed when Smith's medium was supplemented with edamin and inositol and

TABLE 7

INITIATION OF CALLUS FROM SEEDLING SEGMENTS OF WHEAT, RICE AND SORGHUM ON DIFFERENT MEDIA

Mineral salts: (Macro and micro) Modified White's or Smith's (Tables 2A,2B). KI was omitted from some media, given in Table.

Vitamins and glycine: According to Table 2C.

Concentration of supplements (% or ppm): CM (15); edamin (1000); inositol (100); adenine (1); guanine (1); cytidylic acid (1); tyrosine (2); NAA (1 or 5).

Inoculum: 5 mm seedling segments (root or stem).

Period (days): 45

Growth measurements after 45 days (mg): +, 30–40; 2+,40–50; 3+,60–80; 4+,over 100; –,tissue dies

	Mineral salts	KI	DPU	Other supplements	NAA	Callus growth		
						Wheat	Rice	Sorghum
					ppm			
1	White	+	–	–	1			–
2	"	+	–	CM	1			+
3	"	+	+	–	1			++
4	Smith	–	–	CM	1	++		
5	"	–	+	–	1	+++		
6	"	–	+	CM	1	+++		
7	"	–	+	Edamin	1	++		
8	"	–	–	Edamin	1	++		
9	"	–	+	Edamin, inositol	5	++++	++	+++
10	"	–	–	Edamin, inositol	5		++	+++
11	"	+	–	Edamin, inositol, adenine,guanine	5		++	
12	"	+	–	Edamin, inositol, adenine, guanine, cytidylic acid	5		++	
13	"	+	–	Edamin, inositol, adenine, guanine, tyrosine	5		+++	
14	"	–	–	2% starch (instead of sucrose),edamin, inositol	5			+++

NAA added at 5 ppm (Media 9 and 10). Starch instead of sucrose also served as a good carbohydrate source (Medium 14).

b) Long term studies on subculture of wheat, rice and sorghum

The results of long term subculture studies on these three cereals are shown in Table 8. Only a few media were tested with these cultures. Modified Smith's medium with 5 ppm NAA and edamin and inositol was found to permit survival of wheat and sorghum for over 4 years. Addition of DPU had no significant effect on the survival of wheat tissue. The survival of the other tissues on a DPU-free medium was not tested. Sorghum could also be maintained on a medium in which sucrose was replaced by starch, but this experiment was terminated after 20 subcultures.

Rice cultures were routinely maintained for over 3 years on the same medium supplemented with adenine, guanine and cytidylic acid or tyrosine. NAA in all these media was added at 5 ppm on the basis of the results with maize.

Table 9 gives the effect of a few other media which were tested for three subcultures on wheat and sorghum. Wheat tissue grew even when edamin was replaced by casein hydrolyzate or when sucrose was replaced by starch(Media 1 and 2), though the growth was poor. Sorghum tissue also grew when the medium was supplemented with adenine,guanine and cytidylic acid. However these studies were discontinued after three passages.

TABLE 8

EFFECT OF DIFFERENT MEDIA ON GROWTH AND SUBCULTURE OF WHEAT, RICE AND SORGHUM

Mineral salts: (Macro and micro) Modified Smith's (Tables 2A,2B).

Vitamins and glycine: As in Table 2C.

Supplements added to mineral salts and vitamins:(ppm) - edamin (1000); inositol (100); DPU (1); adenine (1); guanine (1); cytidylic acid (1); tyrosine (2); NAA (5).

In medium 6, 2% starch was added instead of sucrose.

Inoculum (mg): 40-50.

Period (days): 45

	DPU	Carbohydrate	Other supplements	Wet weight at last passage	Number of passages	Period of growth
				mg		months
Wheat						
1	-	Sucrose	Edamin, inositol, NAA	532	35	53
2	+	Sucrose	" " "	419	35	53
Rice						
3	+	Sucrose	Edamin, inositol, NAA, adenine, tyrosine, guanine	746	26	38
4	+	Sucrose	Edamin, inositol, NAA, adenine, guanine, cytidylic acid	601	26	38
Sorghum						
5	+	Sucrose	Edamin, inositol NAA	180	30	45
6	+	Starch	" " "	170	28	Experiment terminated after 20 passages

TABLE 9

EFFECT OF A FEW MEDIA ON GROWTH OF WHEAT AND SORGHUM OVER THREE SUBCULTURES

Mineral salts:(Macro and micro) Modified Smith's (Tables 2A,2B)

Vitamins and glycine: As in Table 2C.

Supplements added to mineral salts and vitamins (ppm): edamin (1000); inositol (100); DPU (1); adenine (1); guanine (1), cytidylic acid (1); NAA (5),casein hydrolyzate (1000).

In medium 2, 2% starch was added instead of sucrose.

Inoculum (mg): 40-50.

Period (days): 45

These experiments were studied over three passages

	DPU	Carbohydrate	Other supplements	Wet weight at 3rd subculture
				mg
Wheat				
1	+	Sucrose	Casein hydrolyzate inositol, NAA	133
2	+	Starch	Edamin, inositol, NAA	184
Sorghum				
3	+	Sucrose	Edamin, inositol, adenine, guanine, cytidylic acid	166

SECTION 3

Change of growth rate of the cereals on subculture

It was observed during the course of this work that the tissues were generally slow growing in the initial stages, but on subculture there was a progressive increase in the rate of growth. A record was therefore maintained of the change in the rate of growth of maize, wheat, rice and sorghum over a period of 42 to 52 months. To simplify the presentation each point is given as the ratio of the final to initial fresh weights and represents the average obtained for every four successive subcultures (Fig.2). It will be seen that all four cultures showed a low growth rate initially which increased progressively on subculture. The extent of this increase in growth rate varied with the tissue, being the highest with rice.

The reason for the change in growth rate of these cultures is not known. It may be due to a selection of rapidly growing cells or of cells better adapted to the specific growth medium. The ratio of callus to roots (see Section 4) was not significantly different for any of these cultures as far as could be judged visually at different periods of subculture; the change in growth rate was not due to greater or lesser root formation. (It will be seen in a later chapter that growth of maize and wheat was strikingly increased when they grew as roots in a liquid medium.) Further work is necessary to explain this increase in growth rate with subculture.

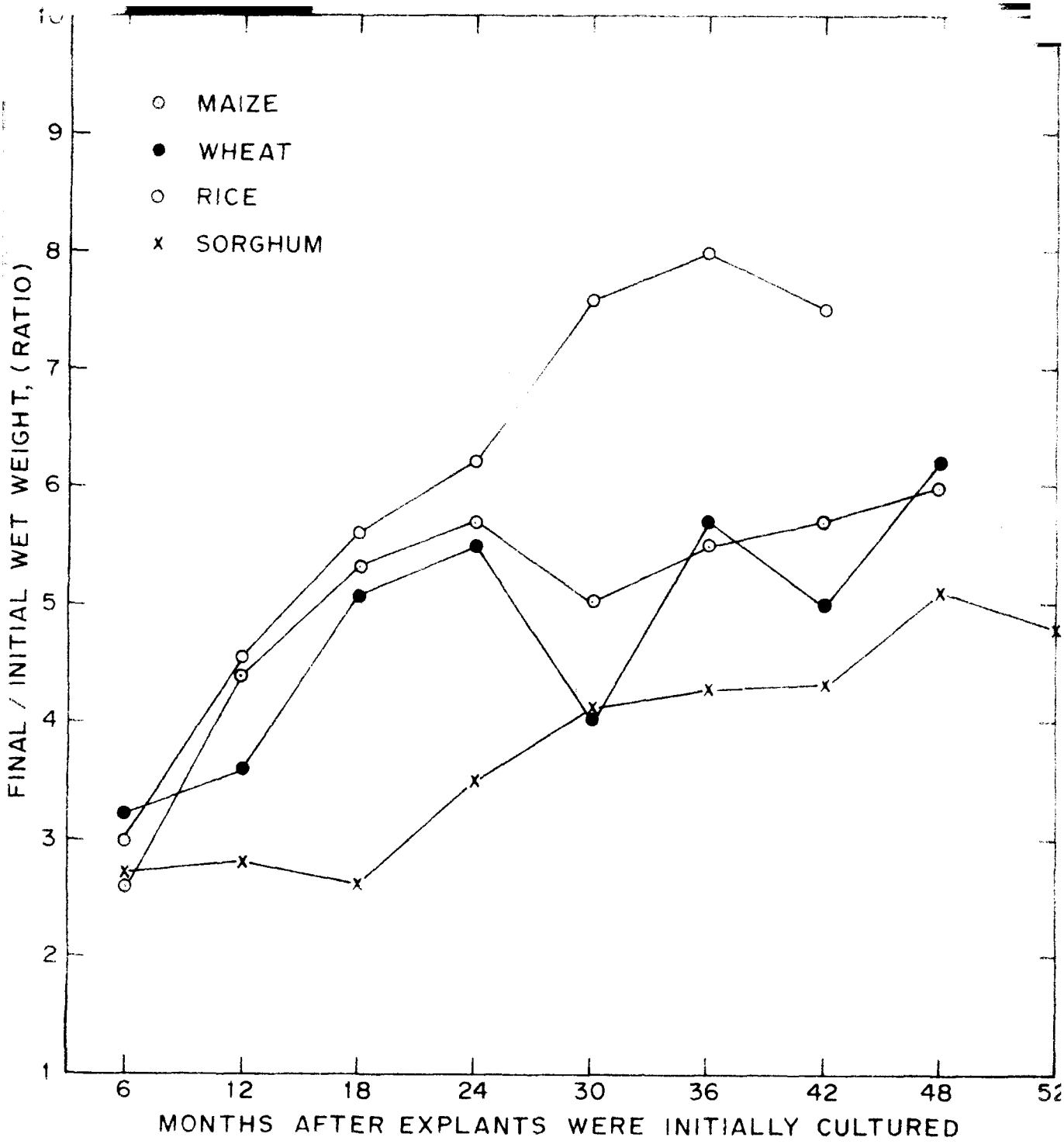


FIG. 2.

CHANGE OF GROWTH RATE OF CEREALS ON SUBCULTURE

MEDIUM FOR MAIZE, WHEAT AND SORGHUM : BM2 , FOR RICE: BM3

EACH POINT IS AVERAGE OF 4 SUCCESSIVE SUBCULTURES

SECTION 4

Appearance of cultures and reproducibility of results

a) Appearance of cultures (Plate 2)

Maize and sorghum cultures grown on agar medium generally consisted of a mixture of callus and roots. Wheat consisted almost entirely of callus with only a few roots in the later stages of growth. Rice showed no root formation. The extent of root formation was markedly dependent on the composition of the medium and the period of culture. The number of roots increased if the cultures were maintained for over 90 days. High concentrations of NAA (5 ppm and over) reduced root formation, whereas low concentrations of auxin favoured root formation. However when NAA was replaced by 2,4-D at 5 ppm, sorghum tissue was observed to grow purely as a callus culture. Maize and wheat tissues were whitish in appearance. Rice cultures soon after initiation grew as a mass of greyish black callus, but on repeated subculture the appearance was white. Sorghum cultures on the other hand consisted of a mixture of pink and white tissue from which a reddish pink pigment diffused into the medium. All the cultured tissues were examined microscopically to confirm that the callus tissue contained only undifferentiated callus cells.

b) Reproducibility of results

Tissue cultures were established from the seedlings of each cereal in four independent experiments with maize and in two experiments with each of the other cereals. The cultures each time grew very poorly during the initial stages, but the growth rate improved markedly on repeated subculture. Using medium BM_2, callus cultures were initiated from 3 different varieties of maize and 2 each of

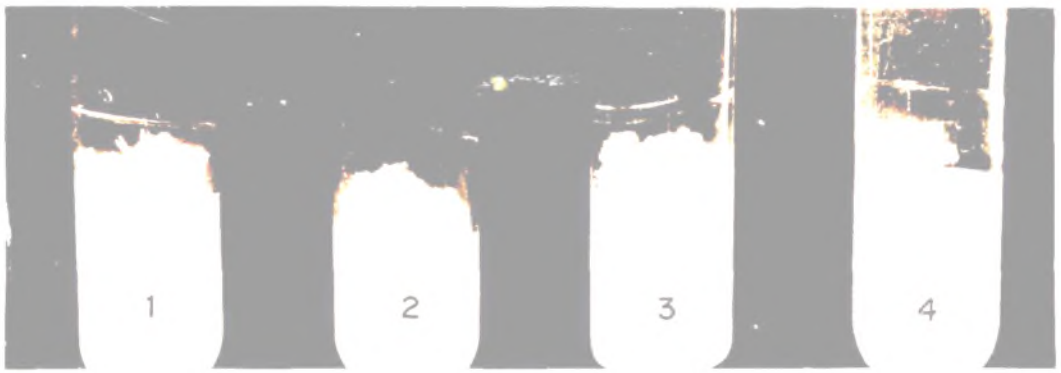


PLATE 2: TISSUE CULTURES OF (1) MAIZE (2) WHEAT (3) RICE AND (4) SORGHUM.

Maize and Wheat were grown on BM_2 medium and Rice on BM_3 medium. Sorghum cultures were grown on BM_2 medium with 2,4-D instead of NAA (cultures 45 days old).

wheat and rice. However, no detailed studies were carried out on these cultures to study their viability on subculture after 3 passages. The method of initiation of callus appeared to be reproducible, but several different seed varieties require to be tested to establish that these methods are widely applicable.

The results of this chapter indicate that the objective of obtaining viable tissue cultures from maize, wheat, rice and sorghum was achieved. The minimum medium for initiation and growth of maize consisted of Smith's or White's medium supplemented with a protein hydrolyzate and NAA. Supplements, such as DPU and inositol, though not essential, greatly stimulated growth of maize. Similar growth requirements were found for wheat and sorghum. Higher NAA (5 ppm) in all cases increased growth greatly. Rice tissue on the other hand grew very well on supplementation of the medium with adenine, guanine and tyrosine. It is surprising that the nutritional requirements of these monocotyledonous cultures are much simpler than those of several dicotyledonous tissues which require cytokinins, coconut milk etc. The failure to grow many of these cultures earlier is apparently not due to the requirement of any new growth factors.

SECTION 5

Discussion

The primary purpose of the present studies was to obtain viable callus cultures from different cereals on a chemically defined medium. This objective has been achieved for maize, wheat, rice and sorghum.

1. Initiation of callus

From the data presented in Table 1 (Introduction) regarding the media used by different authors for growing monocot cultures, it will be observed that the media in general contained mineral salts, vitamins and glycine, an auxin, coconut milk and in many cases yeast extract or casein hydrolyzate.

Similarly for the initiation of callus cultures from maize, a mineral salt medium containing vitamins and NAA and sucrose was the minimum requirement. CM, CSL, DPU and edamin added singly or in combination also stimulated growth and callus formation in maize explants to different degrees. Maximum callus growth was however observed on a high mineral salt-sucrose medium (Smith's) containing higher vitamin and NAA concentrations to which inositol and edamin were added. With 2,4-D and BTOA maize tissue did not grow (Table 4). The results on the initiation of callus from wheat, rice and sorghum also indicated that Smith's medium with sucrose was superior to White's and that very good callus growth could be obtained in the presence of high levels of vitamins and NAA containing the supplements, inositol and edamin with or without DPU. Rice tissue was found to callus better by additional supplementation with adenine, guanine and tyrosine (Table 7). Though callus formation was initially

obtained with rice and sorghum in the presence of CM or DPU or both and with NAA at 1 ppm, the tissue did not survive on subculture. It is not however certain whether the part of the seedling which was used for callus formation also determines the survival of the cultures and not merely the medium. Further work is in progress on this aspect, but will not be dealt with in this thesis since the results are not yet conclusive.

2. Long term subculture

Several media have been used to obtain continuous cultures of somatic tissue of cereals. These media have already been briefly reviewed in the Introduction and will not be discussed here. Recently Mullin (189) obtained callus from a number of monocots and has given the number of passages the tissues have been cultured and the media used for their growth. The medium for initiation of callus for onion, spanish bluebell and day lily was different from the medium which the tissue later needed for its survival. The main supplements used for the different tissues were coconut milk, yeast extract, inositol, malt extract and auxin added in different combinations. The sucrose levels varied from 0.6 to 6 per cent with different tissues. Asparagus was in its 15th passage whereas rye callus died after 13 passages. Spanish bluebell cultures were in their 17th passage.

The effect of subculture on maize tissue (Table 6) was studied in detail by incorporating in the basic media a wide variety of nutrients. An organic nitrogen source edamin or casein hydrolyzate, an auxin and glucose or sucrose added to White's or Smith's mineral salts give sustained growth. The absolute need for organic N was however not established since an earlier series showed growth on

White's basal-DPU-NAA medium. Further work is needed to clarify whether inorganic nitrogen is sufficient for survival of these cultures. Maize tissues can also grow on glucose or starch as carbohydrate sources. DPU was not found to be essential in long term studies with maize tissues, although it was incorporated in most of the media as a result of earlier studies with this growth factor. The growth obtained in the presence of higher levels of the vitamins and glycine was greater than that obtained with these supplements added at a tenth of this level. (The lower concentration is the one used in White's original medium and subsequently by several other workers.) These vitamins and glycine were therefore added at the higher concentration in all further studies. From the overall growth of maize tissue over long periods it could be seen that potassium iodide was not essential. However iodine may be present in agar in sufficient quantity in organic form, and other nutrients may also contain traces of iodine. Several experiments were carried out on the effect of iodine, but the results were inconclusive. Similarly with inositol or adenine and guanine no marked improvement in the overall growth was observed. The tissue survived at two levels of NAA, 1 and 5 ppm, the higher level being superior for overall growth. Supplementation with ammonium nitrate in addition to potassium and calcium nitrates, did not affect survival but gave no increase in growth (Expt.11, Table 6). Since the tissue has been growing on some of the media for over 5 years without diminution in growth, it can definitely be stated that viable cultures of maize have been obtained. The wheat and sorghum tissues (Table 8) are also viable on basal medium supplemented with edamin, inositol and NAA and high vitamins. Rice tissue was found to grow better with adenine, guanine and

cytidylic acid or tyrosine. Wheat and sorghum tissues have been growing for over 4 years and rice for over 3 years (Table 8). In short term experiments it is possible, especially with slow growing tissues, that there is sufficient carry-over of unidentified nutrientw or growth factors to permit growth for a few subcultures. But the four cereal cultures have undergone at least an increase in weight corresponding to about 10^{12} (if 25 subcultures are made with an increase of 3 times in weight at each subculture) and it is improbable that they require any nutrients or growth factors other than those in the respective media. They may also be regarded as viable on subculture in vitro on these media.

4. Change of growth rate on subculture

Street et al (301) studied the effect of subculture on excised wheat roots. The initial high rate of growth declined after the first 14 days of culture and thereafter a low and very variable growth rate was maintained. Lavee and Messer (145) studied the growth of callus cultures of olive over 18 passages for over $2\frac{1}{2}$ years. He found a very rapid drop in growth vigor which continued until the 9th subculture. Then an equilibrium was reached although a very high variability occurred from one subculture to the next.

Figure 3 shows the effect of subculture on maize, wheat, rice and sorghum growing for over 3 years by subculture every 45 days. Initially the growth rates were very poor for all the four cereals. Maize and wheat tissues showed a gradual increase in growth on repeated subculture although considerable variation was observed from one passage to the next. Rice tissue showed a steady rise in growth with every subculture, whereas with sorghum tissue the rise was slower.

Gautheret (85) observed that strains of normal carrot tissue capable of culture in the presence of added IAA gradually changed in their response to this auxin. Kandler (123) also reported similar changes in response to auxin. White (353) found that various strains of tomato roots grew equally well in media containing thiamine plus glycine or thiamine plus pyridoxine. When one of the clones maintained on thiamine plus glycine was transferred to thiamine plus pyridoxine medium, it grew poorly in the first culture period (30 per cent of that in thiamine plus glycine control), but by its third growth period the growth rate had risen to a value of 82 per cent of that in the simultaneous thiamine plus glycine control. The roots apparently required a period of "adaptation" to their new medium. Lavee and Messer (145) found that olive tissues required different growth supplements at different periods of subculture. Chen and Galston (64) also showed that Pelargonium pith cells grown in vitro showed different growth regulator requirements at various stages of development. Almestrand (7) studied the effect of weekly subculture on rye roots. He noticed that some roots became thin as cell divisions stopped and elongation continued. The other types of roots increased in diameter and length and continued to grow for over half a year. Straus (292) also noticed occasional changes in the colour of his maize endosperm tissues from deep purple to white and was able to isolate and maintain them separately. The pigmented tissues had a growth rate of just about half that of non-pigmented tissues.

It may be stated that even after growth for 3 to 5 years in vitro no habituation was observed with the cereal tissues

described in this report. The cultures showed an absolute requirement for auxin even after several years of subculture *in vitro* and in absence of auxin the tissue died after one or two subcultures. Similarly the morphological appearance (varying amounts of callus and roots) also showed no change on subculture. The difference was mainly in the rate of growth on prolonged maintenance *in vitro*.

4. Appearance of cultures

The maize cultures appeared as a mixture of callus and roots in all media. Sorghum cultures grew in most media as a mixture of callus and roots but when NAA was replaced by 2,4-D the culture grew purely as callus. These cultures consisted of a mixture of white and red tissue. The sorghum cultures obtained by Masteller and Holden (163) on medium containing 1 to 5 ppm 2,4-D was found to consist of white, yellow and black callus tissue.

Wheat callus tissue obtained by Trione *et al* (323) consisted primarily of undifferentiated cells. Shimada *et al* (258) observed with wheat tissue that when 2,4-D was added at 1 ppm or higher without other growth substances only callus formation occurred; but when casein hydrolyzate or CM were also added the tissue grew as a mixture of callus and roots. The wheat cultures grown in these studies consisted of callus, but root formation was observed on incubation of these cultures for over 90 days or in the presence of low levels of NAA. The rice tissue grown by Yatazawa *et al* (365) grew only as callus like the rice cultures in the present studies.

CHAPTER III

NUTRITION OF MAIZE, WHEAT, RICE AND SORGHUM TISSUES ON AGAR MEDIA

(Pages 78 - 140)

C O N T E N T S

SECTION 1

Nutrition of maize, wheat, rice and sorghum tissues

After obtaining tissue cultures of maize, wheat, rice and sorghum which were viable *in vitro*, the optimum requirements for the growth of the four cultures were then studied. The nutrition of maize was studied in greater detail than that of the other tissues. The effect of different basal media, nitrogen and carbon sources, auxins, cytokinins, gibberellins, growth retardants and other supplements, and of pH and temperature was tested and the optimum concentrations of nutrients determined in some cases. The experimental conditions regarding the inoculum, medium, period of growth, etc. under which these experiments were performed are given in Chapter I. The experiments described in this and subsequent chapters are not necessarily in chronological order and it should be stressed again that the variations in the final weights of the tissues often grown in identical media may be due to the period for which the cultures had been maintained *in vitro* when the experiment was performed. As has been described in the previous Chapter, the rate of growth of the cultures changed progressively during maintenance. While the results in each Table can be compared, the results of two different Tables even under identical conditions are not necessarily comparable. Controls were therefore run in each experiment and the significance of each experiment has been discussed with reference to the controls in each case. Carryover of nutrients with the inoculum could also have influenced some of the results. For instance in some cases, the tissues showed growth even in the absence of an auxin whereas in others the tissue died. It was not practicable to test for carryover of nutrients by repeated subculture in all cases since this would have necessitated

an unduly large number of experiments over a long period. More uniform results might also be obtained by greater control of the type of inoculum used, by separating the roots from the callus, but this could only be done visually to a limited extent.

In most of the Tables the changes in the composition of the media are given with respect to the BM_2 medium for maize, wheat and sorghum and the BM_3 medium for rice (Table 2D). Both wet and dry weights were determined in most cases but greater importance was given to the dry weight. It was not feasible to determine cell numbers routinely and hence the final dry weight was taken as a measure of growth. The standard error of mean has been discussed earlier in Chapter I and on the basis of those results it has arbitrarily been considered that the results were considered significant only when the growth obtained differed from the control by at least 20 to 25 per cent or more. The standard deviation and significance have not been calculated for the very large number of experiments reported here.

a) Mineral nutrition

Several inorganic solutions have been used for growing plant cell cultures (110, 193, 352). The effect of five different basal media which have been widely used in plant tissue culture, was tested on the growth of the four tissues (Table 10). The iron requirement was supplied in all cases as ferrous-EDTA as it had been shown by Street et al (300) to be a stable chelate which maintains iron availability over a wide pH range. The micronutrient requirements were not studied separately since agar and edamin were added to all media, and may have supplied certain elements in trace amounts. Unless otherwise indicated the concentrations of glycine and vitamins were ten times higher than in White's B in all the media.

On a dry weight basis maize tissue grew best on Smith's medium. The water content of maize tissue grown on Murashige and Skoog's medium was however higher than that on other media as shown by the high wet weight. The optimum medium for wheat was that of Hildebrandt followed by those of Smith and White. On Murashige and Skoog's medium growth was poor. The possibility that wheat tissue requires low salt concentrations needs examination. The growth of rice tissue was maximum on Murashige and Skoog's medium. There was no striking difference in the growth of sorghum tissue on the different media. Most of the subsequent work was however carried out only with Smith's medium for all the tissues in order to avoid a multiplicity of basal media and since it was adequate for all the cultures (Table 10).

TABLE 10

EFFECT OF DIFFERENT MINERAL SALTS MEDIA ON MAIZE, WHEAT, RICE AND SORGHUM

Composition of media:

Macro salts as in Table 2A.

Micro salts: White's micro salts as in Table 2B (White)
Micro salts in other media as in Table 2B (Smith).

Vitamins and glycine: Table 2C.

Other supplements (ppm): For maize, wheat and sorghum, edamin (1000), DPU (1), inositol (100), NAA (5).

For rice adenine (1), guanine (1) and tyrosine (2) together with supplements used for maize etc.

Period (days): 40

Mineral salt media	Maize		Wheat		Rice		Sorghum	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
	mg/tube							
1 White's	389	42	194	15	272	40	115	13
2 Smith's	558	60	181	16	409	53	117	12
3 Murashige & Skoog's	630	46	102	8	656	81	97	10
4 Knop's	478	40	120	10	345	42	162	13
5 Hildebrandt's	489	47	229	21	302	44	92	10

b) Nitrogen

In Smith's medium the main source of nitrogen is calcium and potassium nitrate. The effect of different nitrogen sources is shown in Table 11. Calcium and potassium were supplied as equivalent amounts of the chlorides, and nitrogen was added in inorganic (experiments 1-5) or organic forms (experiments 6-11). All the organic compounds except urea were added at the concentrations given in the Table and not in amounts equivalent to the nitrogen present in Smith's medium. Maize, wheat and sorghum tissues showed markedly higher growth on ammonium nitrate than on nitrate alone (Media 3). Ammonium sulphate and ammonium chloride however gave negligible growth. (The growth observed even on medium containing no nitrogen or with ammonium sulfate and chloride was probably due to a small carry over of nutrient with the inoculum.) Urea, edamin and yeast extract could completely replace inorganic nitrogen in the medium for maize tissue, the growth being of the same order as that obtained with ammonium nitrate. With CM, ME or casein hydrolyzate as the only nitrogen source, the growth obtained was similar to the growth with nitrate alone. Edamin was the best nitrogen source for wheat whereas growth with CM and yeast and malt extracts and casein hydrolyzate was comparable to that with nitrate alone. Though sorghum tissue grew best on edamin and urea, this was less than that on ammonium nitrate. It should however be noted that the total N in experiments 7-11 was variable and not the same as in the other experiments.

Ammonium nitrate concentrations

Since ammonium nitrate gave maximum growth of maize, wheat and sorghum the effect of different concentrations of this salt was determined. In this experiment however (Table 12) ammonium nitrate was

TABLE 11

EFFECT OF DIFFERENT NITROGEN SOURCES ON MAIZE, WHEAT AND SORGHUM

Medium: BM_2

Changes: Edamin, calcium nitrate and potassium nitrate omitted and calcium chloride and potassium chloride added to give equivalent amounts of calcium and potassium. Total nitrogen in experiments 1, 3, 4, 5 and 6 was the same (142 mg/l). Organic nitrogen sources added at following concentrations (%): edamin (0.1), CM (10), YE (0.1); ME (0.1), casein hydrolyzate (0.1).

Period (days): 45

	Nitrogen source	Maize		Wheat		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry
		mg/tube					
1	Nitrate as in Smith's	243	30	334	29	100	17
2	None	177	18	292	24	61	10
3	Ammonium nitrate	598	55	566	46	303	32
4	Ammonium sulphate	180	19	291	21	74	9
5	Ammonium chloride	156	16	179	22	112	14
6	Urea	458	51	176	13	117	16
7	Edamin	435	47	554	43	137	16
8	Coconut milk	225	22	424	26	95	10
9	Yeast extract	477	49	402	28	77	12
10	Malt extract	257	27	348	27	50	7
11	Casein hydrolyzate	278	26	377	29	56	8

TABLE 12

EFFECT OF DIFFERENT AMMONIUM NITRATE CONCENTRATIONS ON MAIZE, WHEAT, RICE AND SORGHUM

Media: BM_2 for maize, wheat and sorghum.

BM_3 for rice.

Changes: Ammonium nitrate added at concentrations specified in Column 2.

Period (days): 40

	Concentration of ammonium nitrate	Maize		Wheat		Rice		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube							
1	0	268	25	286	19	757	86	154	17
2	50	226	21	336	26	810	76	150	16
3	100	459	35	399	33	937	88	156	17
4	250	233	19	428	36	955	88	106	9
5	500	254	20	339	26	1003	98	81	8
6	1000	174	15	346	27	820	83	78	8
7	1500	188	16	285	25	848	84	90	8

added in addition to the nitrate present in Smith's medium and in combination with edamin. Under these conditions 100 ppm was the optimum concentration for maize and 100-250 ppm for wheat. There was no significant effect on the other tissues. It is noteworthy that even at 1500 ppm ammonium nitrate was not markedly toxic to wheat and rice.

Edamin concentrations

From Table 11 it was seen that edamin was suitable as the sole nitrogen source for maize, wheat and sorghum. The effect of different concentrations of edamin was then tested in addition to the nitrate present in Smith's medium. The overall growth with maize in this experiment was comparatively poor, but edamin at 0.05 to 0.1 per cent was stimulatory for all the tissues (Table 13). At a 2 per cent concentration of this protein digest wheat and rice tissues showed growth but the tissue turned black.

Amino acids

Edamin, a lactalbumin hydrolyzate supplied by Sheffield Chemical Company, New York, contains 6-7 per cent amino nitrogen. Since this supplement stimulated the growth of the cereal cultures the effect of different amino acids was tested singly or in groups on maize in order to ascertain whether the growth stimulation was due to a particular amino acid or a group of amino acids. The overall growths in this study were fairly high, being an experiment performed very recently (Table 14). None of the amino acids or groups of amino acids equalled edamin in its effect on maize tissue.

TABLE 13

EFFECT OF EDAMIN CONCENTRATION ON MAIZE, WHEAT, RICE AND SORGHUM

Media: BM_2 for maize, wheat and sorghum.
BM_3 for rice.

Changes: Edamin added at concentrations in Column 2.

Period (days):45

	Edamin concentration	Maize		Wheat		Rice		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
	per cent	mg/tube							
1	0	-	-	519	32	323	39	68	9
2	0.05	230	25	687	40	523	57	63	7
3	0.10	257	26	663	43	694	74	139	17
4	0.25	102	9	481	35	404	50	68	7
5	0.50	121	12	481	36	269	36	81	9
6	1.00	86	5	293	26	288	38	91	10
7	2.00	81	5	219	20	170	25	51	8

TABLE 14

EFFECT OF DIFFERENT AMINO ACIDS ON MAIZE

Medium: BM_2

Changes: Edamin and glycine omitted from the basal medium in all media except 1. Amino acids added alone or in groups at concentrations given in brackets (ppm) in column 2.

Group-I: L-lysine (15.6), L-arginine (8.0), histidine (2.6), DL-methionine (13).

Group-II: Glutamic acid (14), DL-aspartic acid (6), L-asparagine (20).

Group-III: L-valine (13), L-threonine (13), DL-leucine (15.6), DL-isoleucine (10.4), DL-phenylalanine (5) and DL-tryptophan (4).

Added singly: L-tyrosine (2), L-cystine (1), L-glutamine (75), glycine (30).

Period (days): 45

	Amino acid (single or in groups)	Maize	
		Wet	Dry
		mg/tube	
1	Edamin	862	95
2	Group I	613	72
3	Group II	620	76
4	Group III	606	73
5	Groups I and II	378	49
6	Groups I and III	452	56
7	Groups II and III	190	22
8	Groups I,II and III	592	63
9	Tyrosine	396	44
10	Cystine	428	55
11	Glutamine	464	38
12	Glycine	325	36

c) Carbohydrate sources (Table 15)

The effect of different carbon sources, which included sugars, starch, glycerol and sorbitol, was tested on the four tissues. Sucrose was omitted from the basal medium and the carbon source was added at 2% (inositol at 100 ppm was present in the basal medium except in experiment 1). There was no growth in any of the tissues in the absence of a carbon source (Medium 2). Maize grew best on sucrose, maltose and fructose. Starch, sorbitol and glucose were less effective. With lactose, galactose, xylose and glycerol there was very little growth. With wheat and rice tissues optimum growth was obtained with maltose, glucose and sucrose and lesser growth with starch and fructose though in the case of wheat, glycerol also supported growth. The other sources tested had little effect on growth. Sorghum tissue showed good growth on sucrose, starch and glucose and very little on the other compounds. It is noteworthy that all the tissues grew on starch. In long term experiments with maize and sorghum, good growth was observed on starch media even over long periods (Tables 6 and 8, Chapter II).

Sucrose and maltose concentrations

The effect of different concentrations of sucrose and maltose was tested with rice. There was a sharp optimum with sucrose at 2%, whereas with maltose there was good growth at 1-3% (Tables 16 & 17). With a mixture of sucrose and maltose, each added at 1 per cent level, the overall growth was very much higher than with maltose alone at 2 per cent (Medium 7, Table 17).

TABLE 15

EFFECT OF CARBOHYDRATE SOURCES ON MAIZE, WHEAT, RICE AND SORGHUM

Media: BM_2 for maize, wheat and sorghum.

BM_3 for rice.

Changes: Sucrose omitted and replaced by respective carbon source (column 2) at 2%.

Period (days): 45

	Carbohydrate	Maize		Wheat		Rice		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
		mg/tube							
1	(Minus inositol) + sucrose	387	23	333	25	272	25	119	14
2	Nil	81	6	113	7	73	4	-	-
3	Sucrose	576	62	284	23	408	32	136	17
4	Glucose	160	18	375	24	392	28	128	14
5	Fructose	316	37	187	15	198	14	53	9
6	Starch	240	22	173	17	262	12	144	15
7	Maltose	436	38	413	27	567	38	40	4
8	Xylose	125	11	129	9	101	6	48	5
9	Lactose	84	9	127	8	156	9	44	6
10	Galactose	82	6	85	6	95	6	42	7
11	Glycerol	100	10	256	19	81	8	46	5
12	Sorbitol	156	19	138	10	95	7	52	7

TABLE 16

EFFECT OF DIFFERENT CONCENTRATIONS OF SUCROSE ON RICE

Medium: BM_3 with sucrose added at the concentrations given in column 2.

Period (days): 45

	Sucrose concentration	Rice	
		Wet	Dry
	per cent	mg/tube	
1	0	89	6
2	1.0	309	25
3	1.5	546	43
4	2.0	765	69
5	2.5	324	33
6	3.0	275	32

TABLE 17

EFFECT OF MALTOSE CONCENTRATIONS ON RICE

Medium: RM_3

Changes: Sucrose omitted and maltose or sucrose and maltose added at concentration specified in column 2.

Period (days): 45

	Carbohydrate concentration		Rice	
			Wet	Dry
		(per cent)	mg/tube	
1	Maltose	1.0	383	29
2	"	1.5	349	28
3	"	2.0	349	30
4	"	2.5	323	26
5	"	3.0	262	24
6	Maltose	1.5 + sucrose 0.5	205	18
7	Maltose	1.0 + sucrose 1.0	526	46
8	Maltose	0.5 + sucrose 1.5	475	38
9	Sucrose	2.0	400	32

d) Vitamins

Smith's and White's media contain only vitamins B_1 and B_6 and nicotinic acid. The effect of these and other vitamins was then determined by testing the effect of a mixture of vitamins and the effect of omitting one vitamin from each of the media. With maize tissue (Table 18) there was diminution in growth (relative to that obtained when all the vitamins were added) when either ascorbic acid, biotin or thiamine hydrochloride was omitted from the medium. However long term experiments are needed with these cultures to determine which vitamins are essential and which are stimulatory.

Inositol concentration

Inositol was added routinely to all the different media at 100 ppm, but its essentiality or effect was not determined earlier. The next two experiments give the results on the effect of different inositol concentrations at the two levels of NAA of 1 and 5 ppm (Tables 19 and 20). With maize at 1 ppm NAA there was no effect of inositol at low concentrations and inhibition at 50 ppm or higher. Wheat tissues showed stimulation of growth at 50 ppm inositol (Table 19). At 5 ppm NAA, inositol had no significant effect on maize or wheat. The effect of inositol on rice tissue is very marked, this tissue showing progressive growth increases even upto inositol concentrations as high as 500 ppm. However it should be noted that in other experiments inositol showed growth stimulation (experiments 1 and 3, Table 15). As in the case of diphenyl urea, the effect of inositol was not quite reproducible. It was, however, routinely added to the media for all the cultures.

TABLE 18

EFFECT OF SINGLE OMISSION OF VITAMINS ON MAIZE

Medium: BM_2

Changes: In experiments 2-12 vitamins B_1, B_6, nicotinic acid and inositol omitted from BM_2 and a mixture of vitamins added together, or omitting in each case a single vitamin shown in column 2. Concentration of vitamins in the vitamin mixture given in brackets (ppm): ascorbic acid (1), choline HCl (10); B_{12}(0.001), calcium pantothenate (1), biotin (0.01), inositol (100), thiamine (1), niacin (5), pyridoxine HCl (1), riboflavin (0.1).

	Vitamin omitted	Maize	
		Wet	Dry
		mg/tube	
1	Basal media	310	28
2	All vitamins added	695	54
3	Ascorbic acid	273	24
4	Choline chloride	556	41
5	B_{12}	485	37
6	Calcium pantothenate	572	43
7	Biotin	279	22
8	Thiamine hydrochloride	226	20
9	Nicotinic acid	912	68
10	Pyridoxine hydrochloride	632	48
11	Riboflavin	594	48
12	Inositol	580	45

TABLE 19

EFFECT OF INOSITOL CONCENTRATION ON MAIZE AND WHEAT

Media: BM_2

Changes: Concentration of inositol varied, NAA (1 ppm)

Period (days): 45

	Inositol concentration	Maize		Wheat	
		Wet	Dry	Wet	Dry
	ppm	mg/tube			
1	0	375	32	157	15
2	1	389	31	196	18
3	10	393	31	179	18
4	50	264	26	228	20
5	100	238	20	176	14
6	200	210	18	147	11

TABLE 20

EFFECT OF INOSITOL CONCENTRATION ON WHEAT, MAIZE AND RICE

Media: BM_2 for wheat

BM_3 for rice

Changes: Inositol concentrations varied. NAA (5 ppm)

Period (days): 45

	Inositol concentration	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube					
1	0	321	32	294	20	261	28
2	1	253	24	246	18	296	38
3	10	372	34	263	21	391	45
4	50	341	32	218	15	475	54
5	100	373	36	183	13	-	-
6	200	212	21	202	13	-	-
7	500	-	-	-	-	648	69

TABLE 21

EFFECT OF DIFFERENT AMOUNTS OF WHITE'S 'B' MEDIUM ON MAIZE, WHEAT AND RICE

Media: BM_2 for maize and wheat, BM_3 for rice.

Changes: Composition of modified White's 'B' (Table 2C) (ppm): thiamine HCl (1), pyridoxine HCl (1), nicotinic acid (5), glycine (30). These amounts correspond to a level of 1.0 (experiment 4). In other experiments the levels of these supplements were varied as shown in column 2.

Period (days): 45

	Amount of modified White's 'B' Supplements	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
		mg/tube					
1	0	217	22	196	16	86	10
2	0.1	390	35	249	22	142	14
3	0.5	359	45	272	26	653	80
4	1.0	625	80	400	28	276	27
5	1.5	653	80	222	20	86	8
6	2.0	710	82	269	18	84	8

White's 'B' concentration

The vitamin supplement in the original medium according to White contains thiamine hydrochloride (0.1 ppm), pyridoxine hydrochloride (0.1 ppm), nicotinic acid (0.5 ppm) and glycine (3.0 ppm) and is designated as White's 'B' or vitamin solution (though it contains glycine, which is not generally regarded as a vitamin). It should be noted that the level used routinely as shown in Table 2C in the modified White's 'B' solution is 10 times higher than in White's 'B'. The effect of different concentrations of these supplements was determined by increasing the amounts of all these vitamins and glycine simultaneously. They were added at 0.1, 0.5, 1.0, 1.5 and 2 times the levels used routinely (Table 2C). The optimum amount of these supplements for maize and wheat was the quantity routinely added (i.e., 10 times higher than in the original White's medium) and for rice about half this amount (Table 21). There was an appreciable carry over of these nutrients with the inoculum but in spite of this the standard amounts added according to White (0.1 level) was clearly sub-optimum for these tissues.

e) Cytokinins

Coconut milk: Coconut milk has been used extensively in studies on the growth of plant tissues. The results of Chapter II indicated that CM added at 15 per cent concentration was not beneficial for the growth of callus tissues of these cereals. The effect of different concentrations of CM was tested on maize and wheat (with NAA levels at 1 or 5 ppm) and with rice at 5 ppm NAA (Tables 22 and 23). A slight growth stimulation was seen with wheat tissue when CM was added at 1 per cent concentration (volume by volume) to a medium containing 1 ppm NAA whereas it had very little effect on maize. CM at 20 per cent was inhibitory to both tissues. At 5 ppm NAA maize showed only inhibition at all levels of CM, whereas with wheat there was stimulation of growth at 1 to 5 per cent CM. Coconut milk had no stimulatory or inhibitory effect on rice (Table 23).

Diphenyl urea: Diphenyl urea was reported by Shantz and Steward (254) to be one of the active constituents of coconut milk. This substance was therefore tried in all the earlier studies for the isolation of tissue cultures of the cereals and was added at 1 ppm (Chapter II). Though it was found to be necessary for growth of maize in the initial stages, it was observed in subsequent experiments that maize could survive even in the absence of this chemical. In this experiment different concentrations of DPU were tested separately at two different levels of NAA (1 and 5 ppm) (Tables 24 and 25). Maize and wheat tissues did not show any response to DPU at 1 ppm NAA. When the NAA level was 5 ppm there was a stimulation in the growth of maize at 0.001 ppm, of rice at 0.01 ppm and of wheat over a broader concentration range. Owing to the possible carry over of growth factor with the inoculum and the low concentrations which seemed to stimulate growth, more extensive

TABLE 22

EFFECT OF COCONUT MILK CONCENTRATION ON MAIZE & WHEAT

Media: BM_2 for maize and wheat.

Changes: NAA (1 ppm), CM added at different concentrations.

Period (days): 45

	Coconut milk concentration	Maize		Wheat	
		Wet	Dry	Wet	Dry
	%	mg/litre			
1	0	297	22	413	26
2	1	326	25	612	34
3	5	321	23	466	25
4	10	273	20	379	25
5	15	173	14	281	22
6	20	187	15	227	16

TABLE 23

EFFECT OF COCONUT MILK CONCENTRATION ON MAIZE, WHEAT AND RICE

Media: BM_2 for maize and wheat, BM_3 for rice.

Changes: CM added at different concentrations, NAA (5 ppm).

Period (days): 45

	Coconut milk concentration	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
	per cent	mg/tube					
1	0	503	36	287	16	373	40
2	1	339	27	493	28	448	48
3	5	330	23	396	23	314	42
4	10	273	21	281	16	314	40
5	15	262	20	269	15		
6	20	211	18	207	13		

TABLE 24

EFFECT OF DIFFERENT CONCENTRATIONS OF DIPHENYLUREA ON MAIZE AND WHEAT

Media: BM_2 for maize and wheat.

Changes: DPU concentration varied. NAA (1 ppm).

Period (days): 45

	DPU concentration	Maize		Wheat	
		Wet	Dry	Wet	Dry
	ppm	mg/tube			
1	0	653	53	452	33
2	0.001	523	43	386	23
3	0.010	515	43	465	32
4	0.100	568	51	478	32
5	0.500	580	46	458	29
6	1.000	494	41	439	27
7	5.000	284	27	247	19

TABLE 25

EFFECT OF DIFFERENT CONCENTRATIONS OF DIPHENYLUREA ON MAIZE, WHEAT, RICE

Media: BM_2 for maize and wheat; BM_3 for rice.

Changes: DPU concentrations varied, NAA (5 ppm).

Period: (days) 45

	Diphenylurea concentration	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube					
1	0	588	53	407	25	559	61
2	0.001	845	81	500	33	430	47
3	0.010	608	59	590	36	810	77
4	0.100	621	66	480	35	535	57
5	0.500	627	63	469	31	505	36
6	1.000	322	41	466	31	506	35
7	5.000	263	35	173	14	229	27

TABLE 26

EFFECT OF DIFFERENT KINETIN CONCENTRATIONS ON MAIZE AND WHEAT

Media: BM_2 for maize and wheat

Changes: NAA (1 ppm). Kinetin added at different concentrations.

Period (days): 45

	Kinetin concentration	Maize		Wheat	
		Wet	Dry	Wet	Dry
	mg/litre	mg/tube			
1	0	146	14	176	18
2	0.1	146	14	111	9
3	0.2	187	17	101	9
4	0.5	147	14	90	9
5	1.0	112	11	88	8
6	2.0	66	8	77	8

TABLE 27

EFFECT OF DIFFERENT KINETIN CONCENTRATIONS ON MAIZE, WHEAT AND RICE

Media: BM_2 for maize and wheat, BM_3 for rice.

Changes: Kinetin added at different concentrations, NAA (5 ppm).

Period (days): 45.

	Kinetin conetnration	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube					
1	0	288	30	330	30	373	40
2	0.1	213	20	152	9	386	45
3	0.2	368	34	149	12	373	42
4	0.5	303	30	161	9	362	34
5	1.0	198	20	81	7	323	30
6	2.0	174	17	73	7	-	-

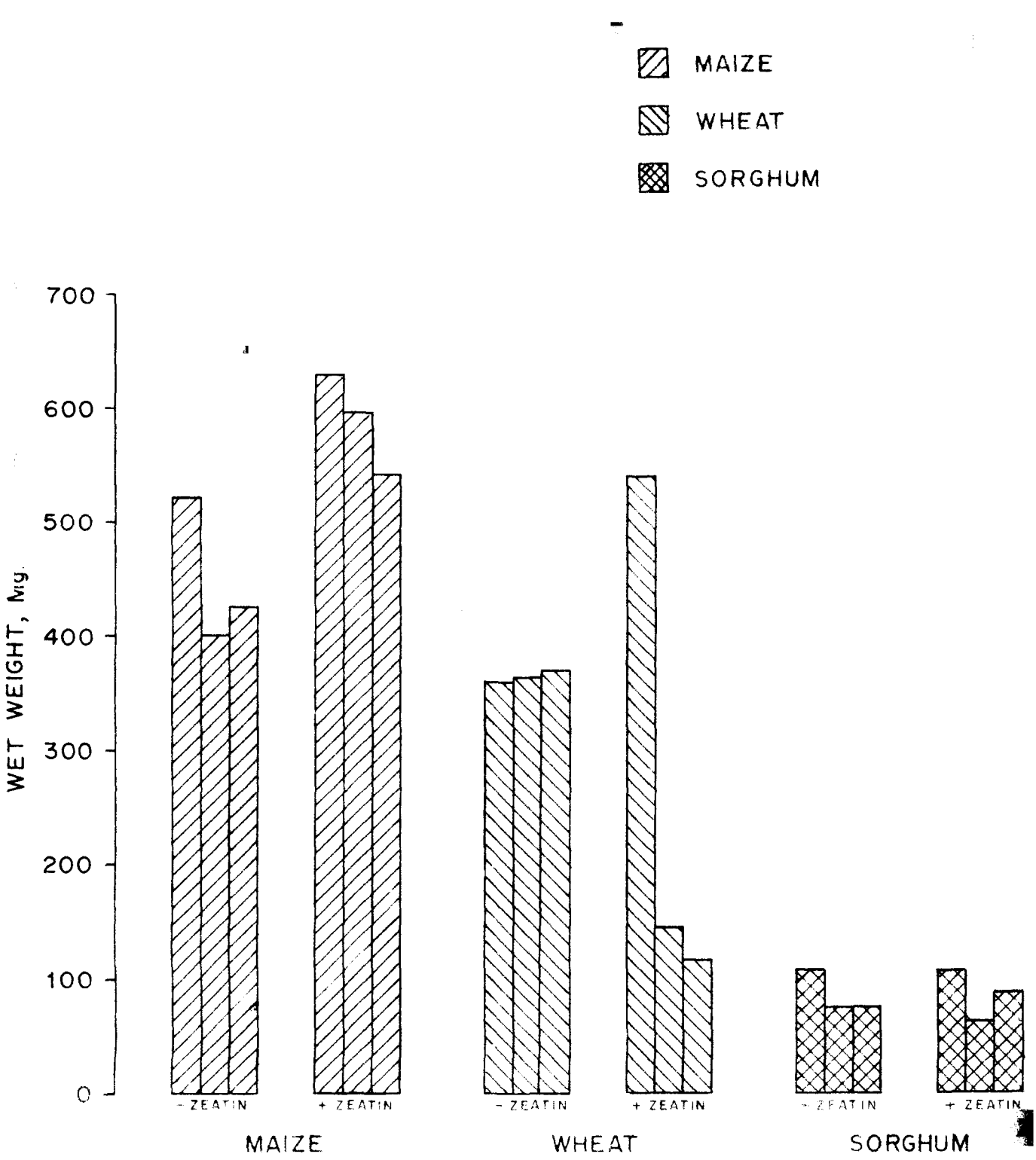


FIG. 3.

EFFECT OF ZEATIN ON GROWTH OF MAIZE, WHEAT AND SORGHUM

(STUDIED OVER 3 PASSAGES)

MEDIUM: BM2 FOR MAIZE, WHEAT AND SORGHUM, (ZEATIN 0·1 µg/l

INOCULUM: MAIZE AND WHEAT: 80–90 Mg. WET

RGHUM: 30–40 Mg. WET

studies are required with tissues grown for many subcultures in the absence of DPU. It would also be necessary to test the effect of DPU at different concentrations and at different auxin concentrations. The results reported here can only be regarded as tentative.

Kinetin: In preliminary experiments, none of the cereals (Chapter II) showed any stimulation by kinetin when it was incorporated in the medium at 1 ppm. The effect of different concentrations of kinetin was tested (Tables 26 and 27) at two levels of NAA. Kinetin had no stimulatory effect on maize, wheat or rice in the presence of either 1 or 5 ppm NAA. It was inhibitory to maize at concentrations higher than 1 ppm and to wheat even at 0.1 ppm.

Zeatin: Since zeatin was isolated from a cereal (170), its effect on maize, wheat and sorghum was tested at 0.1 ug/litre. This experiment was studied over three passages, the same media (with or without zeatin) being used for each successive subculture. Maize tissue showed a slightly higher growth in the first, second and third subcultures as compared with the controls without zeatin (Figure 3). With wheat tissue there was a marked increase in growth in the first subculture, but in the second and third subcultures the growth was less than half that of the controls. This decrease in later subcultures may have been due to an accumulation of zeatin in the tissues. Zeatin had no effect on sorghum culture, the growth in the controls and those with zeatin being nearly the same.

f) Gibberellins

Gibberellic acid (GA_3): The response to gibberellins (mainly gibberellic acid, GA_3) has been studied with a number of tissues (203). No callus tissue till now has been shown to have an absolute growth requirement for gibberellic acid. The effect of GA_3 was tested on maize, wheat and rice at different concentrations (Table 28). All the cultures showed growth inhibition even at low levels of GA_3. However the GA_3 solution was autoclaved along with the media, and further studies would be necessary to determine the effect of cold sterilized gibberellic acid on these cereals, since Murashige and Skoog (193) found that cold sterilized gibberellic acid markedly increased the growth rate of tobacco tissue cultures whereas gibberellic acid autoclaved with the basal medium resulted in little or no growth stimulation.

Different gibberellins: A number of different gibberellins have been isolated from fungal cultures and from higher plant tissues. The eight different gibberellins used in these studies were kindly supplied as a gift by Imperial Chemical Industries, U. K. The different gibberellins were added to the basal media BM_2 at 0.5 ppm (Table 29). Maize tissue showed an inhibition with all the gibberellins, whereas gibberellin A_4 stimulated the growth of wheat tissue. GA_9 was markedly inhibitory to wheat and GA_4 to maize. Further studies however would have to be done to see the effect of cold sterilized gibberellins on the cereals.

TABLE 28

EFFECT OF GIBBERELLIC ACID (GA_3) CONCENTRATION ON MAIZE, WHEAT AND RICE

Media: BM_2 for maize and wheat, BM_3 for rice

Changes: Gibberellic acid (GA_3) added at different concentrations.

Period (days): 40

Gibberellic acid concentration		Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
	ppm			mg/tube			
1	0	380	35	986	65	476	43
2	0.1	319	30	683	50	113	12
3	0.5	199	18	626	45	146	12
4	1.0	175	18	163	16	143	12
5	5.0	223	22	109	10	97	8
6	10.0	156	16	116	12	136	14

TABLE 29

EFFECT OF DIFFERENT GIBBERELLINS ON MAIZE & WHEAT

Media: BM_2 for maize and wheat

Changes: Different gibberellic acids added at 0.5 ppm (GA series).

Period (days): 40

	Gibberellin added	Maize		Wheat	
		Wet	Dry	Wet	Dry
		mg/tube			
1	No gibberellin	532	47	378	29
2	GA_1	144	13	324	16
3	GA_3	278	24	282	19
4	GA_4	83	6	581	39
5	GA_5	181	18	412	27
6	GA_7	201	19	365	24
7	GA_{4+7}	153	13	492	32
8	GA_9	145	11	86	5
9	GA_{13}	152	13	284	15

g) Auxins

The effect of different auxins was tested (Table 30). NAA was omitted from the standard medium and the different auxins were aded at 2 ppm for wheat and maize and at 5 ppm for rice and sorghum. The slight growth observed in the absence of auxin may have been due to a carry over of auxin with the inoculum. In other experiments (not reported here) when the tissue from an auxin-depleted medium was transferred to a similar medium without auxin, the tissue died in all cases showing that auxin is essential for growth of all the cereals. Maize tissue grew well in the presence of IAA, 2,4,5-T and NAA and to a lesser extent with the other auxins. The fact that 2,4-D caused a slight growth stimulation of maize tissue that had undergone many subcultures, but inhibited the earlier explants indicated that the growth factor requirements of this tissue may have changed on subculture. Wheat tissue showed good growth with NAA and to a lesser extent with the other auxins. 2,4-D showed no growth stimulation with wheat. The best auxins for growth of rice were 2,4-D, IBA, alpha- and beta-NAA and to a lesser extent IAA and IPA. With sorghum tissue marked increase in growth was obtained with 2,4-D, the tissue consisting mostly of callus and having a white appearance whereas the tissue grown on NAA consisted of a greater number of roots with a dark red colour (Plate 3). Lesser growth was obtained with the other auxins.

Naphthalene acetic acid concentrations:

The effect of different concentrations of NAA was then tested on maize, wheat, rice and sorghum tissues (Table 31). All the tissues showed tolerance to a wide concentration range of auxin with an optimum at about 5-10 ppm.

TABLE 30

EFFECT OF DIFFERENT AUXINS ON MAIZE, WHEAT, RICE & SORGHUM

Media: BM_2 for maize, wheat and sorghum; BM_3 for rice

Changes: NAA omitted and substituted by the test auxins at 2 ppm for maize and wheat and at 5 ppm for rice and sorghum.

Period (days): 40

	Auxin	Maize		Wheat		Rice		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube							
1	No auxin	86	11	133	10	255	21	44	7
2	α-naphthalene acetic acid	346	36	496	38	673	47	147	20
3	β-naphthalene acetic acid	147	15	183	17	695	50	172	18
4	3-indole acetic acid	423	39	279	26	422	36	117	12
5	3-indole propionic acid	145	15	209	20	385	38	115	13
6	3-indole butyric acid	197	20	241	20	588	53	105	10
7	2,4-dichloro-phenoxyacetic acid	211	22	158	10	712	58	324	34
8	2,4,5-trichloro-phenoxyacetic acid	343	36	207	17	218	18	185	22

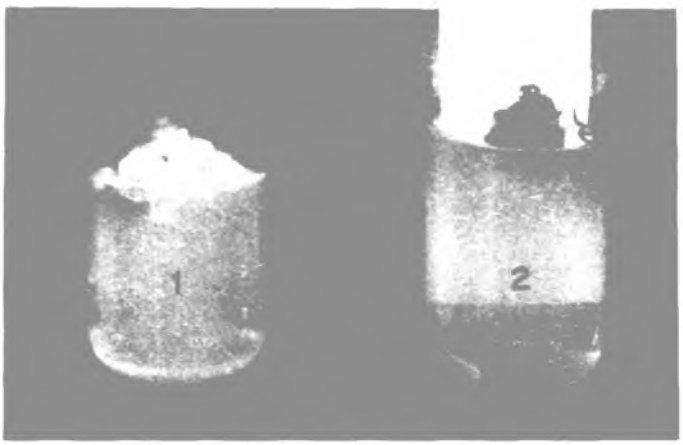


PLATE 3: SORGHUM CULTURES (1) WITH 2,4-D
(2) WITH NAA.

TABLE 31

EFFECT OF NAPHTHALENE ACETIC ACID CONCENTRATION ON MAIZE, WHEAT, RICE AND SORGHUM

Media: BM_2 for maize, wheat and sorghum, BM_3 for rice

Changes: NAA added at different concentrations

Period (days): 40

	α-Naphthalene acetic acid	Maize		Wheat		Rice		Sorghum	
		Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
	ppm	mg/tube							
1	0	86	11	280	23	164	18	61	9
2	0.1	126	10	389	29	181	20	80	12
3	1.0	226	21	415	30	175	20	83	10
4	3.0	-	-	385	30	-	-	110	11
5	5.0	230	21	469	35	245	31	163	20
6	7.0	-	-	480	37	-	-	-	-
7	10.0	253	24	578	40	204	25	75	12
8	15.0	-	-	-	-	186	21	78	12
9	20.0	330	28	353	27	101	15	70	10
10	30.0	-	-	194	16	-	-	-	-
11	40.0	-	-	135	10	-	-	-	-

h) Growth retardants

Cathey (59) used the term "growth retardant" for all chemicals that slow cell division and cell elongation in shoot tissues and regulate plant height physiologically. Although many growth retardants are known, in these studies only two were tested (Table 32) CCC and AMO-1618 which were incorporated into the basal medium for maize at 2 concentrations - 0.1 and 1.0 ppm (Table 32). There was no significant effect of the compounds at the lower concentration but at 1 ppm both of them markedly inhibited growth. An interesting observation in this case was that maize tissue which normally grows as a mixture of callus and roots showed a suppression of root formation in the presence of either of these compounds.

i) Abscisic acid

Abscisic acid is a naturally occurring growth factor showing a multiplicity of inhibitory and other effects (2, 168). The effect of abscisic acid was studied on maize, wheat and sorghum. Maize tissue showed a 50 per cent inhibition when abscisic acid was added at 0.1 ppm and almost complete inhibition at 1 ppm. Wheat and sorghum also showed inhibition at the concentration of abscisic acid tested (0.1 ppm). In order to study the synergistic effect, if any, between abscisic acid and kinetin, the latter compound was added to the medium at 0.2 ppm and abscisic acid at 0.1 ppm either singly or together. Both kinetin and abscisic acid were inhibitory when added singly, but there was no lessening of inhibition when they were added together. Further studies with varying concentrations of kinetin and abscisic acid will be necessary to extend these preliminary observations. An interesting effect in the presence of abscisic acid was a reduction in the ratio of root to callus with maize, wheat and sorghum, root growth being suppressed to a greater extent. Plate 4 shows the effect of abscisic acid on maize tissue.

TABLE 32

EFFECT OF GROWTH RETARDANTS ON MAIZE

Medium: BM_2

Changes: Retardant added at 2 concentrations

Period (days): 42

	Growth retardant added	Concentration	Maize	
			Wet	Dry
		ppm	mg/tube	
1	None	-	335	30
2	CCC	0.1	421	34
3	CCC	1.0	128	10
4	AMO-1618	0.1	432	36
5	AMO-1618	1.0	139	11

TABLE 33

EFFECT OF ABSCISIC ACID AND KINETIN ON MAIZE, WHEAT AND SORGHUM

Medium: BM_2

Changes: Abscisic acid and kinetin added at the concentrations shown below.

Period (days): 40

	Kinetin	Abscisic acid	Maize		Wheat		Sorghum	
			Wet	Dry	Wet	Dry	Wet	Dry
	ppm	ppm	mg/tube					
1	-	-	321	30	468	29	177	17
2	-	0.1	177	15	226	20	125	13
3	-	1.0	86	7				
4	0.2	-	166	15				
5	0.2	0.1	162	13				

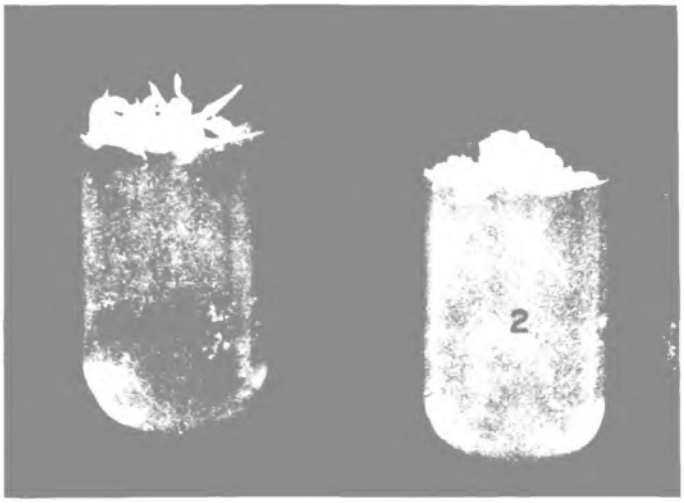


PLATE 4: MAIZE CULTURES (1) WITHOUT ABSCISIC ACID (2) WITH ABSCISIC ACID.

j) Nucleotide bases

Since nucleotide bases appeared to stimulate rice tissue in preliminary experiments, adenine, guanine and/or cytidylic acid were added to all media for growing rice though it was not definitely established whether they were essential for this tissue for subculture over long periods. In this experiment adenine, guanine, uracil, thymidine and cytidylic acid were added singly or together to basal media to test their effect on maize, wheat and rice (Table 34).(Thymidine and CMP were added as the nucleoside and nucleotide respectively and the others as the bases only because of their availability in quantity as those compounds at the time of the test.) Growth stimulation was observed with maize with CMP or adenine, but there was inhibition or no stimulation with mixtures of the compounds. Uracil was inhibitory. With wheat tissue growth increase was observed with all the compounds added singly and with a mixture of adenine, guanine, uracil and cytidylic acid. Though rice tissue showed no significant increase in growth with any of the nucleotide bases added either singly or combined, their presence in the medium did not in any way inhibit growth. However the inoculum for rice was grown on a medium containing nucleotides. The effect of different concentrations of the nucleotides singly or in combination was however not tested and it is possible that the concentration of mixed nucleotides was excessive.

k) Temperature

Plant cultures are generally incubated at a temperature, usually in the range 22–30°C. The effect of three temperatures was tested on maize and better growth was obtained at 28° and 30° than at 25° (Table 35).

TABLE 34

EFFECT OF NUCLEOTIDE BASES ON MAIZE, WHEAT AND RICE

Media: BM_2 for maize and wheat, BM_3 for rice

Changes: Nucleotide bases added at 1 ppm in BM_2 for maize and wheat. For rice, adenine and guanine were omitted from BM_3 medium and the nucleotide bases added alone or combined as in column 2.

Period (days): 45

	Nucleotide base	Maize		Wheat		Rice	
		Wet	Dry	Wet	Dry	Wet	Dry
		mg/tube					
1	No base	209	18	288	17	668	55
2	Adenine	268	24	538	35	672	56
3	Guanine	261	20	488	29	558	45
4	Uracil	64	6	527	32	650	53
5	Cytidylic acid	395	32	368	23	541	49
6	Thymidine	105	10	375	22	709	55
7	Adenine + guanine + uracil + cytidylic acid	152	15	637	37	-	-
8	Adenine + guanine + uracil + cytidylic acid + thymidine	134	12	383	22	-	-
9	Adenine + guanine + uracil	-	-	-	-	678	58

TABLE 35

EFFECT OF TEMPERATURE ON MAIZE

Medium: BM_2

Incubated at 3 different temperatures.

Period (days): 45

	Temperature	Maize	
		Wet	Dry
	°C	mg/tube	
1	25	322	25
2	28	366	41
3	30	374	37

TABLE 36

EFFECT OF HYDROGEN ION CONCENTRATION ON MAIZE & WHEAT

Media: BM_2

Changes: pH of the medium varied

Period (days): 45

	pH	Maize		Wheat	
		Wet	Dry	Wet	Dry
		mg/tube			
1	4	791	47	567	34
2	5	622	63	552	35
3	6	733	59	382	26
4	7	668	56	510	35
5	8	691	57	467	28

l) Hydrogen ion concentration

In all the experiments on the effect of different growth factors on the cereals, the pH of the medium was adjusted to 5.5 to 6.0. To find the optimum pH for maize and wheat, these tissues were grown on media over the pH range of 4.0 to 8.0 (Table 36). Surprisingly both maize and wheat tissues had a very wide pH tolerance and growth was good at all the pH values tested between 4.0 and 8.0. The change in pH during growth was however not tested.

m) Inoculum sizes

Table 37 shows the effect of varying the inoculum size on the final growth of maize. The results of this experiment are represented both by wet and dry weights and by the ratio of the final wet weight to the inoculum wet weight. With inocula of 50, 150 and 250 mg fresh tissue the final ratio is nearly the same, i.e., 4.4 to 4.7. Further work is needed to establish whether the higher ratio of 6.4 obtained with a 100 mg inoculum is of significance. As with many other plant tissues, with very small inocula there was no growth of tissue even after a long period. But no attempt was made to determine the minimum inoculum size required for growth. The main object of this experiment was to establish that the inocula generally used were sufficient to permit growth, and variation of inoculum size within definite limits did not materially affect the experiment provided that all inocula in each set were nearly the same.

n) Agar concentration

Agar is added to different media to serve as a base on which the tissue rests during its growth and to prevent the tissue from

TABLE 37

EFFECT OF DIFFERENT SIZES OF INOCULUM ON MAIZE

Medium: BM_2.

Inoculum sizes varied.

Period (days): 45

	Inoculum size	Maize		Ratio of final wet weight / inoculum
		Wet	Dry	
	mg	mg/tube		
1	50	222	18	4.4
2	100	640	45	6.4
3	150	667	47	4.4
4	200	747	54	3.7
5	250	1171	85	4.7

TABLE 38

EFFECT OF DIFFERENT AGAR CONCENTRATIONS ON MAIZE & WHEAT

Basal media: BM_2

Agar concentration varied.

Period (days): 45

	Agar concentration	Maize		Wheat	
		Wet	Dry	Wet	Dry
	%	mg/tube			
1	0.6	535	39	266	15
2	0.7	573	45	274	16
3	0.8	733	59	382	25
4	0.9	528	44	633	43
5	1.0	388	47	695	48

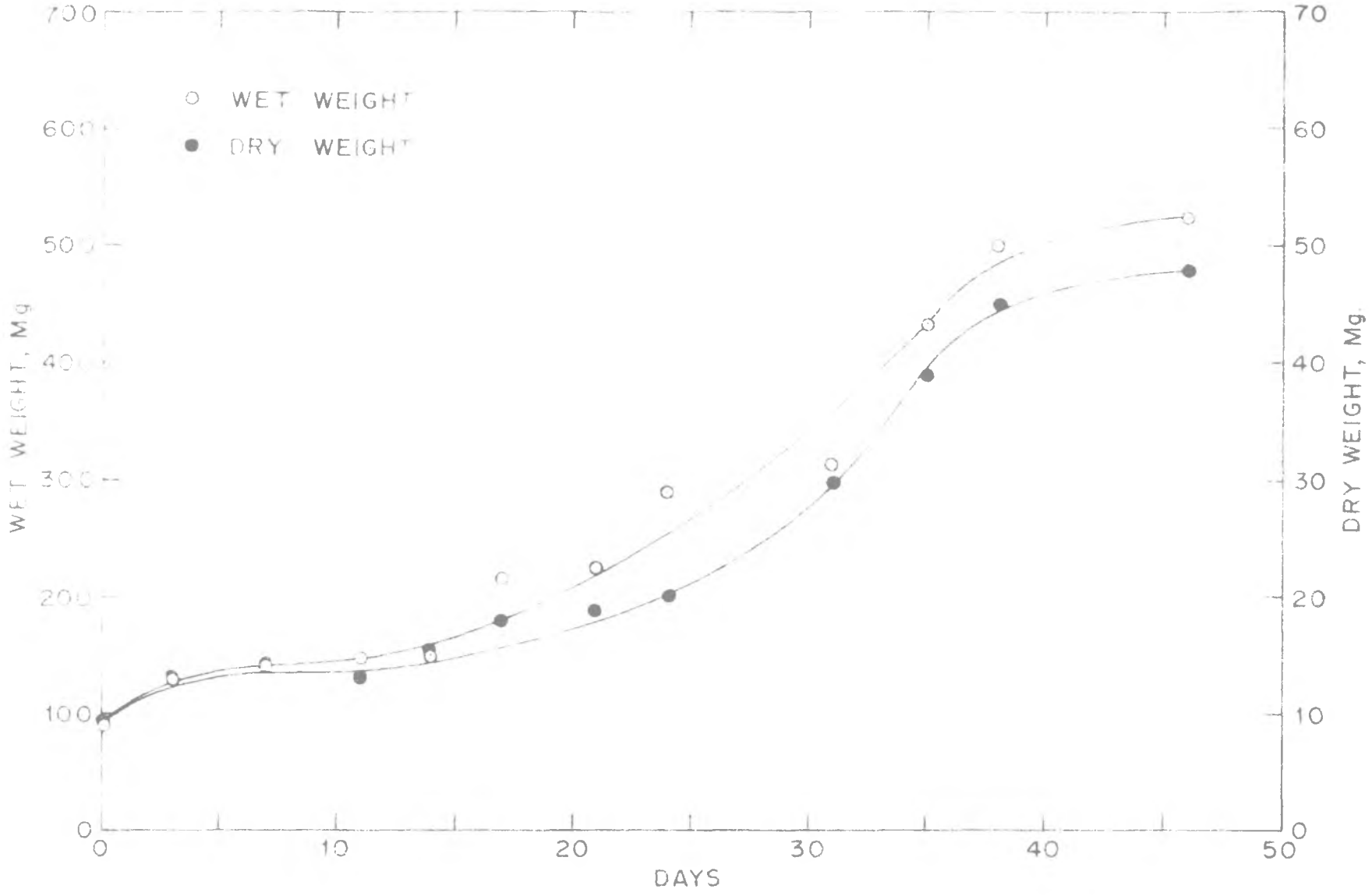


FIG. 4.

GROWTH CURVE OF MAIZE

MEDIUM: BM2.

INOCULUM: 80–90 Mg. WET

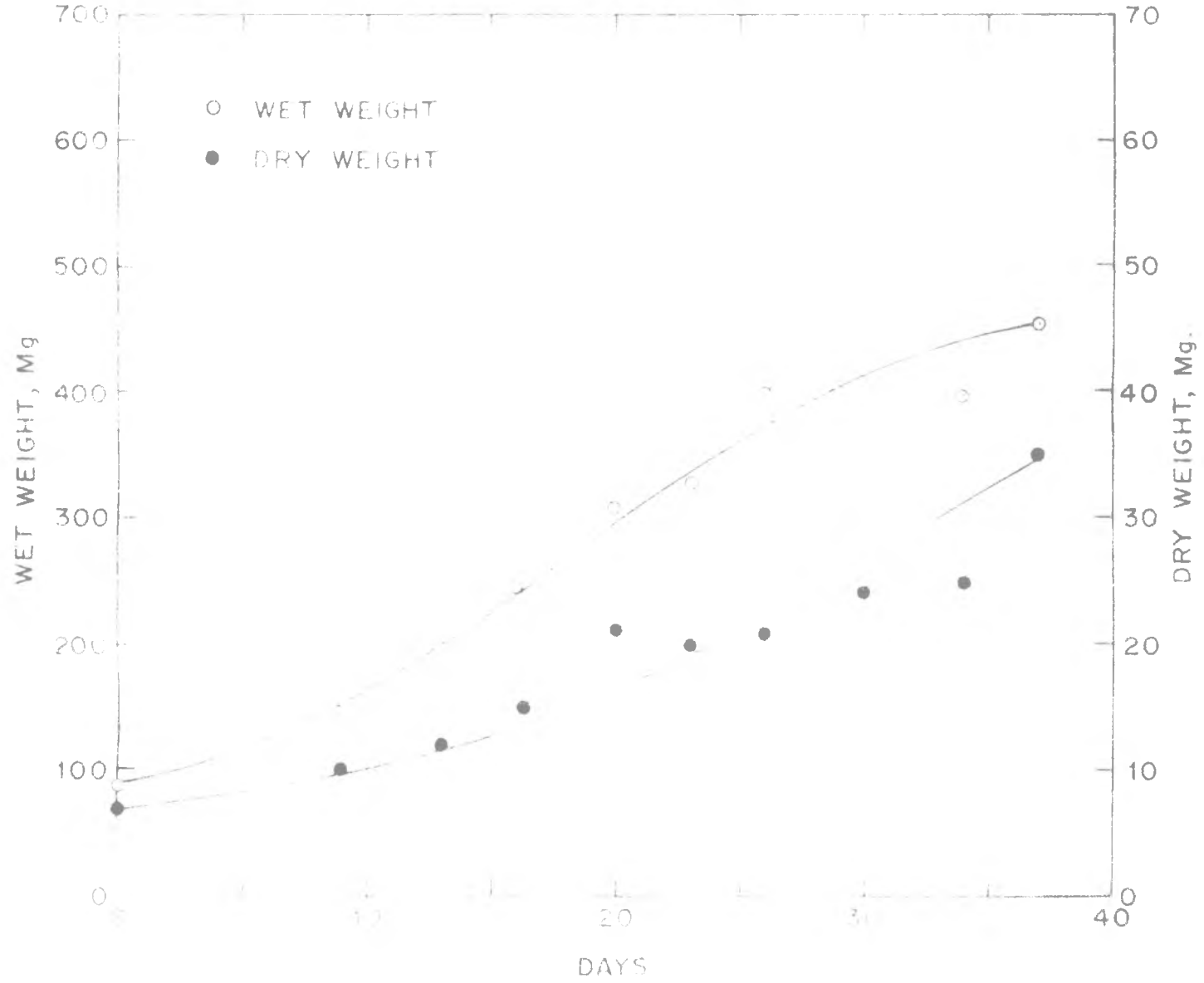
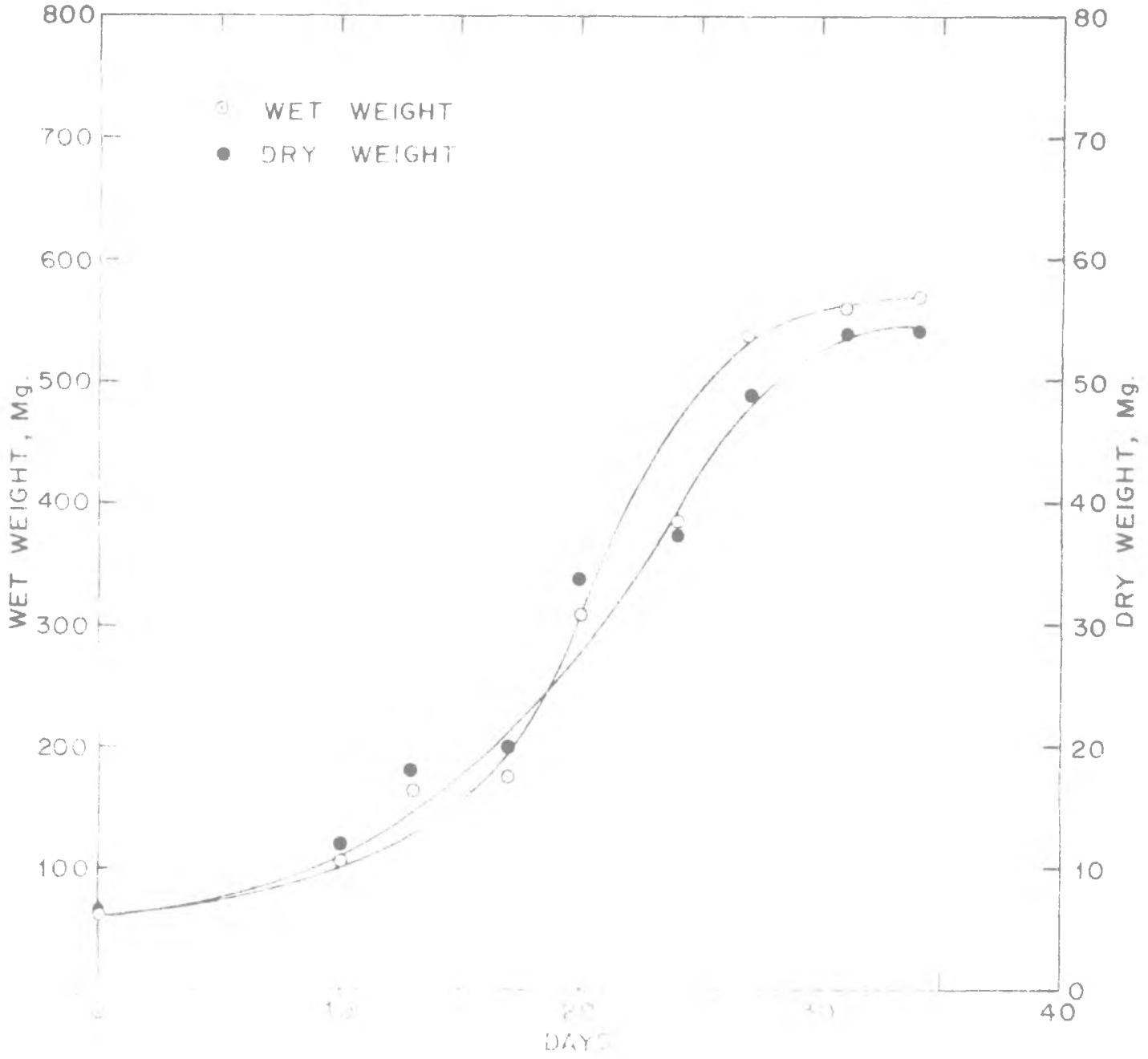


FIG. 5

GROWTH CURVE OF WHEAT

MEDIUM : BM 2

INOCULUM : 80 - 90 Mg WET


WET WEIGHT
DRY WEIGHT
WET WEIGHT, Mg.
DRY WEIGHT, Mg.
800
700
600
500
400
300
200
100
80
70
60
50
40
30
20
10
0
40
DAYS

getting submerged in the medium. If the concentration of agar is too low the tissue does not remain on the surface and is submerged, and if the agar concentration is too high it may affect the diffusion of nutrients to the tissue. Maize grows well over a wide range of agar concentrations (0.6 to 1.0%) whereas wheat shows diminution of growth below 0.9%(Table 38).

o) Growth curves

The growth curves of maize, wheat and rice are shown in Figures 4, 5 and 6. Maize shows a lag period of about 10 days, slow growth during the next 10 days, a logarithmic phase between 25 and 40 days and then a stationary phase. The wet and dry weights run more or less parallel between the 20th and the 45th day, but in the first 12 days the water content of the tissues is less. Wheat tissue (Figure 5) shows a similar lag phase, but there is a less marked difference between the initial slow and subsequent rapid growth as compared with maize. The water content of wheat was higher than that of maize. Rice tissue (Figure 6) also shows a lag period of ten days, a period of slow growth for about 10 days and a period of rapid growth during the next 5 days. There was no marked change in the water content of rice at different stages of growth.

SECTION 2

Discussion

Mineral composition

The mineral composition of the medium plays a very important role in the growth of different plant tissues and organs. Heller (103) noted that plant tissues are very sensitive to mineral deficiencies. White's nutrient solution (352) designed originally for excised root cultures was also employed successfully in the cultivation of various tissues from numerous species (89). Gautheret's medium (82) was devised by combining a twice diluted Knop's (131) macronutrient solution with a slightly modified micronutrient solution (22). Hildebrandt *et al* (110) employed the triangulation technique to improve on White's medium for the cultivation of specific tissues. Murashige and Skoog (193) described a medium containing relatively high concentration of salts, mainly potassium, nitrogen and phosphorus. Smith's medium contains K, N and P at levels intermediate between those of White and of Murashige and Skoog. Morel and Wetmore (187) grew tissue cultures from the monocot *Amorphophallus rivieri* on Gautheret's medium (84). Straus and La Rue (295) used a White's modified nutrient solution by substituting Nitsch's trace elements (207) for those used by White (352) for callus cultures from maize endosperm tissue. Nishi *et al* (206) used Linsmaier and Skoog's medium (150) for obtaining and growing callus from rice. Webster (343) found that when EDTA was added to Heller's nutrient medium with various supplements, callus growth of oats and onions was very good.

Maize tissue showed highest dry weight increases on Smith's medium, although on Murashige and Skoog's medium, the fresh weight

increases were maximum. Murashige and Skoog's medium was the best for rice, and Hildebrandt's for wheat. The five different basal media which were tested differ however in several components. No definite conclusions can be drawn for instance from the data in Table 10 about the specific mineral nutrient or nutrients which cause higher growth of rice on Murashige and Skoog's medium or of maize on Smith's medium. It should however be noted that maize tissue for instance can be maintained for long periods (Table 6, Chapter II) either on modified White's or Smith's medium. Though both media are adequate for prolonged maintenance it would require a very large number of experiments to determine the optimum concentration of each of the macro elements. It is also not known to what extent the micro nutrients which may be present in different quantities in different media contribute to the observed effects on growth. The results reported in Chapter IV however show that nitrogen, phosphorus, iron and sucrose are unlikely to be limiting for the growth of these tissues under the conditions of these experiments.

Nitrogen sources

Inorganic sources of nitrogen for plant tissue cultures can be supplied in the form of nitrates (103,234,352) which have been found to support the growth of a wide range of tissues. However it was found that tobacco and fraxinus cultures showed better growth when the medium contained ammonium nitrate together with another nitrate source (193, 361).

The effect of ammonium salts added as the sole source of nitrogen was tested on maize, wheat and sorghum and it was found that ammonium nitrate was superior to nitrate as the sole N source (Table 11). Since nitrates are reduced to ammonia it is not clear why a combination of

ammonia and nitrate is superior to nitrate and why ammonium sulfate or chloride fail to support growth.

Complex organic sources of nitrogen rich in amino acids such as yeast and malt extracts, casein hydrolyzate, peptone, tomato juice, edamin and some plant extracts have been used extensively in studies on plant tissue cultures. White (349) obtained growth of tomato root tips on media containing yeast extract. Since then yeast extract has been used for cultures of fern (271) pollen cultures of Gingko biloba (324), and for a number of other tissues. Malt extract has been used for the vigorous and continuous growth of Pinus strobus (151) for spruce tissue (272) and for Vigna catjang callus cultures (160). Straus and La Rue (295) used casein hydrolyzate, tomato juice or YE for their maize endosperm cultures. Murashige and Skoog (193) found that an enzymatic digest of lactalbumin, edamin, greatly stimulated growth of tobacco callus cultures. It was also emphasized that the growth promoting activity of coconut milk fractions isolated by them was greatly enhanced in the presence of casein hydrolyzate (253).

The results with maize, wheat and sorghum (Table 11) suggest that these tissues can grow equally well on nitrate or on an organic nitrogen source. Urea was also effective for promoting good growth of maize and sorghum tissues, though not equally good for wheat. Urea was found to be a good nitrogen source for spruce callus (151) and red clover excised roots (102) respectively. However it would be necessary to study the effect of the organic compounds as sole nitrogen sources on subculture to draw definite conclusions regarding their usefulness for maintenance of these cultures.

Murashige and Skoog (193) and later Wolter and Skoog (361) studied the optimum requirement of ammonium nitrate for tobacco and

fraximus cultures and found 1650 mg/l and 50 mg/l respectively to be the best levels.

Maize tissue has an optimum requirement of 100 mg/litre, wheat of 100-250 mg/litre whereas there was no definite stimulation of rice and sorghum by ammonium nitrate. The effect of ammonium nitrate concentrations on the cereals was however tested on Smith's media containing potassium nitrate and edamin (Table 12).

Edamin contains 12.5 per cent total nitrogen of which 52 per cent is amino nitrogen, the major amino acids being valine, leucine, lysine, isoleucine, threonine, arginine, histidine, tryptophane, phenylalanine and methionine, the other amino acids being present in small amounts (Technical data of Sheffield Chemical, New York). When different amino acids were tested for maize singly or in groups (Table 14) it was seen that in no case was growth as good as when edamin was added to Smith's medium containing inorganic nitrate.

Alanine, glycine, arginine, glutamic acid, aspartic acid or asparagine could support the growth of sunflower crown gall tissues although the individual amino acids were inferior to nitrate (235). Nickell(194) & Nickell and Burkholder (199) found that aspartic acid enhanced the growth of sorrel virus tumor tissue when simultaneously supplied with nitrate. Glutamic acid and arginine were reported to increase the growth of cultured asparagus stem tips (77). Jerusalem artichoke tuber tissue grew as well with alanine, γ-amino butyric acid, glutamic acid, aspartic acid, glutamine or urea as with nitrate (209). Arginine, aspartic acid and glutamic acid were found to be the most important amino acids for the growth of sugarcane (200).

Edamin was found to be a suitable supplement for the growth of the four cereal cultures. A level of 0.1% was optimum for the growth of the tissues. The long-term studies reported in Chapter II also show that edamin supplemented media were adequate for the indefinite maintenance of these cultures. But further studies are needed, preferably over several subcultures, to determine the minimum requirements of amino acids for survival and optimum growth of these plant tissues. It is of interest that most of the monocotyledonous plant tissues seem to grow best on a medium supplemented with a protein hydrolyzate, yeast or malt extract or other complex nitrogenous source.

Carbohydrate sources

The carbohydrate requirements of carrot cultures were studied by Gautheret (83, 86) who examined the ability of various sugars to support their growth. Most tissues grow best when supplied with sucrose, glucose or laevulose (104,107,108,199). Galactose has in most cases proved ineffective although for tissues of Vinca rosea (107,108) and Sequoia sempervirens (14) it is reported to be a satisfactory carbon source. Sequoia tissue also grew well on mannose. Hildebrandt and Riker (107,108) have reported the utilization of dextrin, pectins and soluble starch by tissues of Vinca rosea and Chrysanthemum frutescens cultures. Maize endosperm (295) and Acer saccharum tissues (166) utilize starch as a sole carbon source. Acer cultures could also grow on raffinose or maltose. Sugarcane cell suspensions grew best on raffinose followed by sucrose and starch (201). Yatazawa et al (365) working with callus tissues from rice found glucose slightly superior to fructose, sucrose and maltose. Nickell and Burkholder (199) tested the effect of sorbitol, inositol and mannitol as sole carbohydrate sources and found that these compounds could not maintain growth of any of the tissues tested.

Gautheret (87), Hildebrant and Riker (107) found that glycerol could support growth of some tissues.

Sucrose was a good carbon source for all the cereal cultures. Glucose was also a good substrate for wheat, rice and sorghum and to a lesser extent for maize. Starch gave less growth but was utilized by all the cultures. Fructose and maltose were well utilized by all the cultures except sorghum. Maize grew well on sorbitol and wheat on glycerol (Table 15). Sucrose at 2 per cent level in the media was used for growing barley and wheat cultures (79) and for rye callus tissue (63). Mullin (189) added sucrose at 4 to 6 per cent level to the media for growing different monocot cultures. Risser and White (236) found 5 per cent to be the optimum concentration of sucrose for spruce tumor cultures.

The optimum concentration of sucrose for rice tissue was 2 per cent and the optimum maltose concentration was 1 to 3 per cent (Tables 16 and 17). The optimum concentration of sucrose was tested for the other cultures but the results were not reproducible, possibly due to carryover of carbohydrate with the inoculum.

Vitamins

White (349) obtained continued growth of excised tomato roots on a medium containing 0.01 per cent yeast extract and later (350) showed that the yeast extract could be replaced by thiamine. Robbins and Schmidt (241,242,243) reported that a mixture of pyridoxine and thiamine would permit culture of excised tomato roots and found that none of the other B-vitamins could enhance the growth rate further. Bonner and Devirian (34) examined the vitamin requirements of excised roots of ten other species and found that for full growth all required the presence of thiamine in the medium, carrot roots required pyridoxine

also, while sunflower and datura roots required pyridoxine, nicotinic acid and thiamine. Since then most media formulations contained only two or three of these vitamins (103,355). There are some media in which the other vitamins, p-amino benzoic acid, ascorbic acid, biotin, choline, cyanocobalamin, folic acid, inositol, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine are added (149, 347). Reinert and White (234) included ten vitamins in their medium and found B_{12} and biotin necessary for good growth of three out of four strains of Picea glauca tissue. Steinhart et al (272) included choline, inositol and thiamine in their media for growing spruce tissue cultures. Morel (183) found pantothenic acid to be essential for the growth of hawthorn. McClary (167) reported that excised roots of maize synthesized significant quantities of thiamine. Similar results were also obtained by Street (297). Bonner (30,31) found that roots of flax, white clover and tomato, also synthesized biotin and riboflavin, Murashige and Skoog (193) obtained excellent growth of tobacco callus when 100 ppm inositol was also added to their basal media. Straus (291) in his work on maize endosperm tissues incorporated a vitamin solution containing thiamine, nicotinic acid, calcium pantothenate, biotin, inositol, pryidoxine and folic acid in the basal medium. Calcium pantothenate, inositol, vitamin B_{12}, glycine, folic acid, nicotinic acid and thiamine were added to White's basal media in combination with casein hydrolyzate for callus cultures of normal and diseased Pennisetum typhoides tissue (315). Gamborg and Eveleigh's (79) medium contained nicotinic acid, thiamine, pyridoxine and inositol for the growth of wheat and barley cultures. Spruce tissues grew well on a medium containing thiamine, inositol, nicotinic and ascorbic acids (236). These studies were made by omitting single vitamins from the 10 vitamins added to the medium. It should

however be noted that in most cases no definite evidence exists for the essentiality of the vitamins added to the medium. It is not even known for instance whether vitamins such as the cyanocobalamin are required at all for plant tissues.

The effect of vitamins on maize was also tested, omitting one vitamin in each experiment (Table 18). The results were inconclusive though they show a possible stimulatory effect of ascorbic acid, thiamine and biotin on growth. Since there was also stimulation when nicotinic acid was omitted, more detailed studies with varying amounts of these vitamins are needed, especially after a few subcultures to determine which vitamins are essential and which are stimulatory to these cultures.

Staba (268) and Lin and Staba (149) used inositol at concentrations higher than those used by Murashige and Skoog (193) to grow digitalis and mint Fraxinus callus cultures had an absolute requirement for inositol, the growth response being observed at 1 ppm or above (361). In its absence the tissue turned necrotic. Risser and White (235) added inositol at 100 ppm for spruce cultures. Linsmaier and Skoog (150) studied the effect of inositol with different levels of thiamine and found that inositol at 100 ppm was optimum when thiamine was added at 25 ug/litre. For barley and wheat (79) and rye cultures (189) 100 ppm inositol was added to the medium. For the growth of Pennisetum typhoides cultures 300 ppm inositol was added (315).

Inositol had no effect on growth of maize and wheat at NAA levels of 1 or 5 ppm (Tables 19 and 20). However inositol had a marked effect on the growth of rice at all the concentrations from 1 to 500 ppm. The results obtained with inositol in these and other experiments, which will not be discussed in detail, were erratic and not quite reproducible.

Inositol was routinely added at 100 ppm in view of earlier data but no unequivocal requirement or stimulation by it could be demonstrated.

Wolter and Skoog (361) studied the effect of different concentrations of pyridoxine on fraxinus cultures and found 0.05 ppm to be the optimum and to be essential for the growth of this culture. Linsmaier and Skoog (150) also tried the effect of increasing the levels of thiamine, pyridoxine, nicotinic acid and glycine together and found no significant influence on the fresh and dry weight yields, though improved growth vigor was noticeable in treatments with the higher levels. The tissues supplied with 4X and 5X levels of vitamins were more glistening, white and compact than the others. Their studies also revealed that thiamine alone accounted for the full effect of thiamine, pyridoxine, nicotinic acid and glycine when one or more of the four specified substances was omitted from the medium. Nicotinic acid and pyridoxine at 1 ppm and thiamine at 10 ppm were added for growing barley and wheat tissues (79).

The studies with the cereal cultures showed a definite increase in growth when the levels of White's B components were simultaneously increased to 5 to 10 times the concentration used by White and most other workers (Table 21). More detailed studies will be necessary to find out whether the growth increase at higher levels of White's B was due to the combined effect of all four substances or to the effect of a single substance.

Coconut milk

In 1941, van Overbeek *et al* (218) succeeded in growing immature datura embryos in culture by including the liquid endosperm of *Cocos nucifera*, coconut milk, in their culture media. When fragments of

carrot root phloem were cultured in the presence of IAA but without CM the tissues did not grow (50). After this initial work CM was shown to stimulate cell division in other cultured tissues and has been used widely as a supplement for several tissues (66,195,258,276). The concentrations of CM used varied from 1 to 20 per cent. Norstog (213) cultured very small barley embryos on a medium containing 90 per cent CM and White's medium (352). Straus (292) studied the effect of CM on maize endosperm tissue grown in vitro and found that it had no appreciable effect on its growth. A similar effect was also noticed on excised endosperm cultures of corn (320) although 18 per cent CM added to Gautheret's medium (89) was most favourable for culturing meristem tissues. Shimada et al (258) obtained best growth of wheat callus on media containing 10 per cent CM. Nickell (197) also supplemented the medium with 18 per cent CM for growing cultures from internodal parenchyma tissue of several sugarcane varieties.

In the present studies (Table 22) 1 per cent CM showed a slight stimulation with wheat and inhibition at higher concentrations especially at higher NAA concentration. CM had no significant effect on rice cultures.

Diphenylurea concentration

After its demonstration in CM (254), Strong (303) found that DPU had slight and variable cytokinin activity. Bruce and Zwar (40) tested the cytokinin activity of a number of substituted ureas and thioureas and found that all the ureas tested were less active than kinetin and its derivatives. Kinetin was found to be four times as active as the most active urea derivative. Carew and Schwarting (55) observed that DPU was capable of supporting moderate growth of rye embryo callus when used in combination with casein hydrolyzate and 2,4-D. Mascarenhas et al (160)

reported that maize callus tissue had an absolute requirement for DPU for survival. These results were based on maize callus tissue which were transferred through 10 passages. However later results indicated that the requirement of DPU for maize callus cultures was lost on sub-culture as maize was capable of surviving even in the absence of this growth factor.

DPU had no stimulatory effect on maize and wheat tissues when NAA was added at 1 ppm. At 5 ppm NAA there was a stimulation in the growth of maize tissue at 0.001 ppm DPU, rice at 0.01 ppm DPU and wheat over a broader concentration range of 0.001 to 1.0 ppm DPU (Tables 24 and 25). These results require to be confirmed and extended over a larger number of subcultures at lower DPU concentrations.

Kinetin concentration

The literature on the effect of kinetin on plant cultures is very extensive. It was observed (58) that oat callus induction and growth was relatively unaffected by lower kinetin concentrations, whereas higher levels suppressed callus induction. Shimada et al (258) studied the effect of different concentrations of kinetin from 0.01 to 2.0 ppm and found that it did not exert any influence (stimulative or inhibitory) on callus growth of wheat. Tiwari and Arya (315) added kinetin at 0.1 ppm as a supplement for induction and growth of callus from normal and diseased Pennisetum typhoides tissue. In these studies the effect of kinetin on cereal culture was found to be negligible or inhibitory. It is either not required for these cultures or is synthesized by them; or a different cytokinin is the physiologically active form for these cultures (Tables 26,27).

Zeatin

Miller (169,171) was able to detect zeatin at concentrations of about 5 x 10^{-11} M by an assay system using soybean callus tissue. Letham (147) and Miller and Witham (175) found zeatin to be much more active than kinetin in inducing cell division in carrot root and soybean callus tissue cultures. Miller (172) isolated substantial quantities of a cytokinin similar to zeatin from maize - "golden cross bantam". Since the maize variety used in these studies was "Golden Bantam", the effect of zeatin on its growth and on the growth of wheat and sorghum was tested (Figure 3).

Maize tissue showed a slight stimulation with zeatin, the growth being more than that in the controls in all the 3 subcultures. With wheat tissue there was a marked growth stimulation in the first subculture but in the second and third subcultures the inhibition was more than 50 per cent as compared to the controls without zeatin. Zeatin showed no inhibitory or stimulating effect on sorghum.

Gibberellins

Nickell and Tulecke (203) studied the effect of gibberellic acid (GA_3, 10 ppm) on the growth of many plant tissue cultures. With some tissues growth inhibition was observed, whereas with others a slight growth stimulation resulted. Murashige and Skoog (193) observed that cold sterilized gibberellic acid added to the medium resulted in an increased growth rate of tobacco tissue cultures. 1 ppm appeared to be the optimal concentration although even at higher concentrations of 100 ppm there was no growth inhibition. The gibberellin effect at the lower concentrations in these experiments was only on fresh weight. Optimum growth of fraxinus callus cultures was obtained with 100 ppm

gibberellic acid (361). The response started at 5 ppm and high levels (300 ppm) were inhibitory. Established cultures of corn endosperm cultures grown on the yeast extract medium did not respond to gibberellic acid (310). Similar results were also obtained with rye embryo callus cultures (54).

Maize, wheat and rice cultures showed an inhibition with gibberellic acid (GA_3) at concentrations of 0.1 ppm to 10 ppm. The inhibition was very marked in the case of rice callus cultures (Table 28).

Van Overbeek et al (219) suggested that the numbering of the gibberellins at least in the 'A' series (i.e., $GA_{i-\infty}$) be restricted to those isolated from plants or other natural sources. The best known of the gibberellins and commercially produced by fermentation from fungal cultures is gibberellic acid (GA_3). Several other gibberellins are known (221) which differ slightly in chemical structure from GA_3 but vastly from GA_3 and from one another in biological activity (37).

The results with maize and wheat (Table 29) show inhibition by all compounds especially GA_4 of maize growth and by GA_9 of wheat and stimulation of wheat by GA_4.

Auxins

As early as 1938 Went and Thimann (344) realised that the growing regions of plants synthesize minute quantities of several growth regulatory materials which migrate to adjacent cells, where they control cell elongation and several other processes in plants. Of these substances, known as auxins, the naturally occurring compound is indole 3-acetic acid (125). Many synthetic auxins have also been described (338) some of which have biological activities more potent than that of IAA. In tissue

cultures the native auxin IAA is often replaced by 2,4-D or NAA (296). For the culture of most normal callus tissues, auxin is an essential supplement to the basic medium containing inorganic ions and sugar. Examples of such tissues are cited by Nobecourt (212), Gautheret (84, 89) and Morel (184). Carew and Schwarting (54) observed that 2,4-D was superior to IAA for growing rye embryo callus. Tamaoki and Ullstrup (310) found that the established cultures of corn endosperm on the yeast extract medium showed no response to IAA, NAA or 2,4-D at the three concentrations at which they were tested. Mullin (189) used either NAA or 2,4-D in the media for culturing the different monocots. Yatazawa et al (365) observed that the concentration of IAA or NAA required to develop rice callus was ten fold higher than for 2,4-D.

Auxins are undoubtedly essential for the four cereal cultures as shown by their failure to survive on subculture in the absence of auxin. In fact, auxin is the only plant hormone required by these cultures. The results of Table 30 show that IAA or NAA are the best for maize, NAA for wheat, 2,4-D, IBA and 1 or 2-NAA for rice and 2,4-D for sorghum (Table 30).

IAA at a concentration of 50 ppm induced callus formation of wheat, whereas the concentration of 2,4-D that induced a similar response in 3 varieties of wheat, varied from 1.0 to 10 ppm in combination with kinetin(258). Tiwari and Arya (315) studied the effect of varying concentrations of 2,4-D on the growth of normal and diseased Pennisetum typhoides tissue cultures and found 9 ppm to be the optimum level for both the cultures. 2,4-D at 6 ppm was added into the basal medium for callus cultures of wheat and barley (79) and different varieties of sugarcane (197). Oat callus induction and growth were very sensitive to auxin concentration the level of IAA for callus induction being much

higher than that of 2,4-D (58). Webster (343) obtained callus formation and growth of oat tissue on media containing NAA at 25 ppm.

The effect of different NAA concentrations was tested on maize, wheat, rice and sorghum (Table 31) and as can be seen from the Table, maize, wheat and rice tissues withstand fairly high auxin concentrations, 15 ppm or over. The optimum auxin concentration was about 5-10 ppm. These results and those in Chapter IV suggest that cereal cultures possibly differ from dicotyledonous cultures in showing a requirement of or tolerance for higher auxin concentrations than the latter. Further work with a greater number of monocot and dicot cultures would be of interest to determine whether this generalization is valid at least for several of these cultures.

Growth retardants

The effects of plant growth retardants like CCC, phosfon (2,4-dichlorobenzyltributyl phosphonium chloride), B-995 (N-dimethylamino succinamic acid) and AMO-1618 on the aerial portions of plants have been the subject of many studies (59,60,159). Though the growth retardants in most of these studies were applied via the root system, there are very few reports on the effects of these substances on the growth and metabolism of roots or of callus cultures. Inhibition of root growth in barley and rice seedlings by CCC has been reported (127,232). Tung and Raghavan (331) studied the effects of various growth retarding chemicals on the growth of excised roots of Dolichos lablab in sterile culture and found that application of a range of concentrations of CCC, phosfon and B-995 inhibited increase in length of the roots by reducing the frequency of cell division with little or no effect on cell elongation.

The studies with maize (Table 32) showed marked inhibition by CCC and AMO-1618 at 1 ppm with a greater inhibitory effect on roots than on callus as noted visually.

Abscisic acid

Addicott and Lyon (2) identified abscisic acid as a naturally occurring plant growth regulating substance which acted as an inhibitor in various biological systems (168) and counteracted responses induced by other plant hormones (78,314).

Abscisic acid inhibited maize, wheat and sorghum tissues at 0.1 ppm (Table 33). A 10 times higher concentration of this growth substance completely inhibited maize tissue.

Blumenfeld and Gazit (28) studied the interaction of kinetin and abscisic acid in the growth of soybean callus at different levels of kinetin and abscisic acid and found that abscisic acid at 10 ppm acts as an inhibitor with low kinetin levels. This inhibition was reversed when the kinetin level in the nutrient media was raised. Van Overbeek (220) also showed that abscisic acid and kinetin are antagonistic to each other in their effect on the growth of Lemna minor. Aspinal et al (11) also noted a stimulating effect of abscisic acid and of gibberellic acid (GA_4 + GA_7) in promoting cucumber hypocotyl growth. Kinetin was unable to reverse the effect of abscisic acid in the case of maize but the results of Blumenfeld and Gazit (28) suggest the need for testing these two compounds at different concentrations.

Nucleotide bases

Tamaoki and Ullstrup (310) found that the addition of adenine to the basal salt medium had no effect on callus formation from excised corn endosperm. A similar negative result was obtained for rye embryo callus (54). Murashige and Skoog (193) observed that in some cases tobacco tissue cultures gave upto nearly 50 per cent increase in fresh weight when "Braun's supplements" (CMP 200 ppm, GMP 200 ppm, L-asparagine 500 ppm and L-glutamine 500 ppm) were added to the basal medium. Risser and White (236) studied the effect of different concentrations of adenine sulphate on spruce tumor cells but found that it did not enhance the growth of the tissue at any concentration.

A slight growth increase was obtained when maize tissue was grown in the presence of cytidylic acid or adenine and an inhibition was observed with uracil. Wheat tissue growth was greatly enhanced in the presence of most of the nucleotides added alone or in combination. The overall growth with rice was neither stimulated nor inhibited by any of the bases tested (Table 34).

Temperature

Most plant tissues which have been studied were found to have an optimum temperature for growth between 23° and 33°C (89). Optimum growth of spruce tissue was obtained however in the light or in darkness at a temperature of 20°C (358). The growth obtained at 25°C was significantly lower. Shimada et al (258) incubated tissue cultures of wheat at 25°C whereas Gamborg and Eveleigh (79) studied the methods for culturing barley and wheat tissues at 27°C. In this laboratory the temperature of the chamber used for growing tissue cultures was maintained at 25°C$\pm$1° with illumination by diffuse light (5 foot candles)

for about 4 hours per day. The studies at 28° and 30°C were carried out in incubators in darkness. The growth was better at the higher temperatures but no definite comparisons can, however, be made of the results of Table 35 since there was a difference in illumination.

Hydrogen ion concentration

Hildebrandt et al (109) studied the effect of the hydrogen ion concentration of the medium on the growth of tobacco and sunflower tissue and found that at pH 3.0 the tissues were adversely affected while optimum growth was obtained between pH 4.5 and 6.0. White and Risser (358) found that spruce tissues have a pH optimum between 5.5 and 6.5. Straus and La Rue (295) found that an initial pH of 6.1 to 7.0 provides for optimum growth of maize endosperm callus cultures whereas Tiwari and Arya (315) obtained best growth of normal and diseased Pennisetum typhoides tissue cultures at pH 6.0. Sheat et al (256) and Hannay et al (101) adopting ferric-EDTA as the source of iron and by the use of sparingly soluble calcium salts as buffers to maintain the pH of the medium at any level within the range 4.0 to 7.5, found tne effectiveness of nitrate for active growth of excised roots over the whole pH range whereas active growth with ammonium as sole source of nitrogen occurs only over a narrow pH range close to neutrality.

Both maize and wheat tissues (Table 36) grew well over a wide pH range from 4.0 to 8.0 and have a broad pH optimum. It will be seen from a later Chapter with liquid media that there was no appreciable change in the pH of the medium during the growth period.

Inoculum sizes

Nitsch (208) observed that when two or more fragments of Jerusalem artichoke tissue were placed in the same culture flask, the

growth of each of the fragments was greater than that of comparable fragments placed singly in culture flasks. He suggested that some factors were released by the explants which stimulated their growth. On the contrary large initial inocula were reported to have a smaller relative growth rate (49,52). Syono and Furuya (307) observed that the lag phase was longer with small inocula than with large inocula in the case of tobacco callus in liquid cultures.

With maize tissue (Table 37) the ratio of final wet weight to initial wet inoculum was the same with inocula of 50, 150 and 200 and 250 mg except for a higher growth rate with 100 mg which requires confirmation. However it must be emphasized that this is true only over the range of inoculum weights which was tested. In the nutritional requirement studies which have been discussed earlier, the inocula used were fairly large (50 mg or more) since with smaller inocula growth was very poor and erratic. The nutritional requirements of smaller inocula may be more complex and other substances may be necessary for growth.

Agar concentrations

White and Risser (358) studied the effect of different agar concentrations ranging from 0.125 per cent to 2.0 per cent on spruce cultures. At 0.125 and 0.25 per cent concentration the tissue tended to get submerged and growth was poor. Two per cent agar also gave poor results. The results obtained with 0.5 and 1.0 per cent were the best, there being no statistical difference between the two

A very small range of agar concentrations between 0.6 and 1.0 per cent was tested for their effect on the growth of maize and wheat tissues (Table 38). The optimum for maize was between 0.7 to 1.0 per cent

whereas wheat tissues showed maximum growth at 0.9 to 1.0 per cent agar.

Growth curve studies

Tissue cultures resemble bacteria etc. in their growth curves and show an initial lag phase followed by a period of rapid growth, a logarithmic phase and then a levelling off of growth. Straus and La Rue (295) found that maize endosperm tissue reached maximum growth by the twentieth day, there being a lag period for 8 days before growth entered the exponential phase. Murashige and Skoog (193) in studying the effect of tobacco leaf extract on tobacco pith tissue also found an initial lag period of 8 days which was followed by a period of rapid growth till about the thirtieth day. The typically sigmoid curve obtained in growth studies on tissues was also obtained with carrot explants (274), with spruce tissues (358) and with Acer pseudoplatanus cultures (166).

Maize, wheat and rice tissue cultures showed similar sigmoid growth with an initial lag phase, a period of exponential growth and a stationary phase. The duration of the three growth phases however varied slightly for the three tissues. The water content of maize and wheat tissues increased with age, whereas no marked change in the water content was observed with rice tissue (Figures 4, 5 and 6).

CHAPTER IV

STUDIES ON MAIZE AND WHEAT TISSUES IN SHAKE FLASKS

C O N T E N T S

SECTION 1

Comparison of growth on agar and liquid media

The isolation of callus cultures of maize, wheat, rice and sorghum and their nutritional requirements on media solidified with agar were described in the previous chapters. As mentioned in Chapter II, Section 4, maize, wheat and sorghum cultures on agar media grew as a mixture of callus and roots, the proportion of callus to roots being dependent on the composition of the medium and the period for which the cultures were incubated, root formation being greatly increased on incubation for 3 months or more. When maize tissue (40 days old) grown on agar medium (BM_2) was transferred to liquid medium of the same composition and agitated on a rotary shaker at 120 to 180 rpm the tissue was found to differentiate into a mass of roots with a number of laterals (Plates 5 and 6). Both wheat and sorghum tissues also grew in liquid media under shake flask conditions giving rise to roots. When rice tissue from agar was transferred into shake flasks, root formation did not take place and the tissue did not grow satisfactorily.

Maize tissue inoculated on BM_2 agar medium grew to a final weight of about 400 mg wet (30 mg dry) from an inoculum of 80-90 mg wet. This corresponded to a 4 to 5 fold increase within 40-45 days. In shake flasks 200-250 mg maize tissue increased to about 3000 to 3500 mg wet (260-300 mg dry). This meant a 12 to 17 fold increase within 18 to 20 days. The overall growth in liquid media is markedly higher than in solid media in a much shorter time. Wheat and sorghum cultures in liquid media however grew at a much slower rate than maize. The growth of wheat was about 2000-2500 mg in 30 days and of sorghum

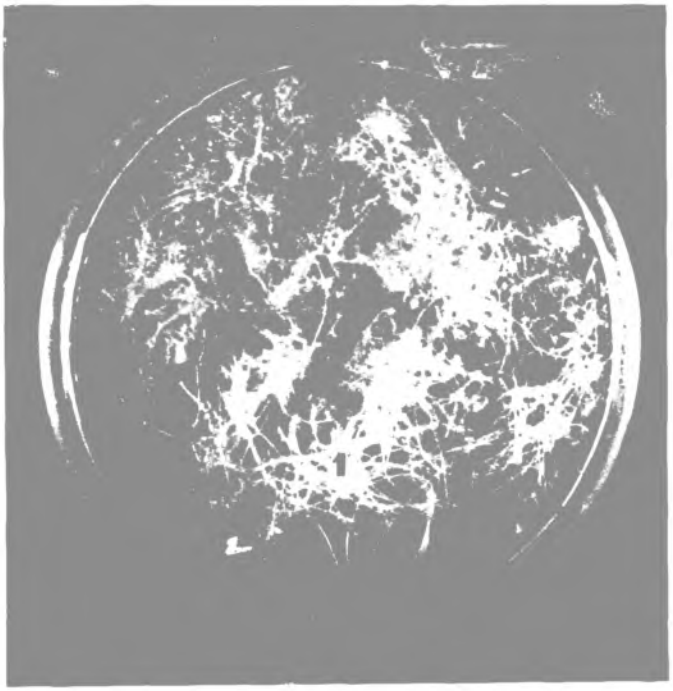


PLATE 5: MAIZE CULTURES GROWN ON BM_2 MEDIUM

(1) SHAKE FLASKS (18 DAYS OLD)

(2) AGAR (45 DAYS OLD)

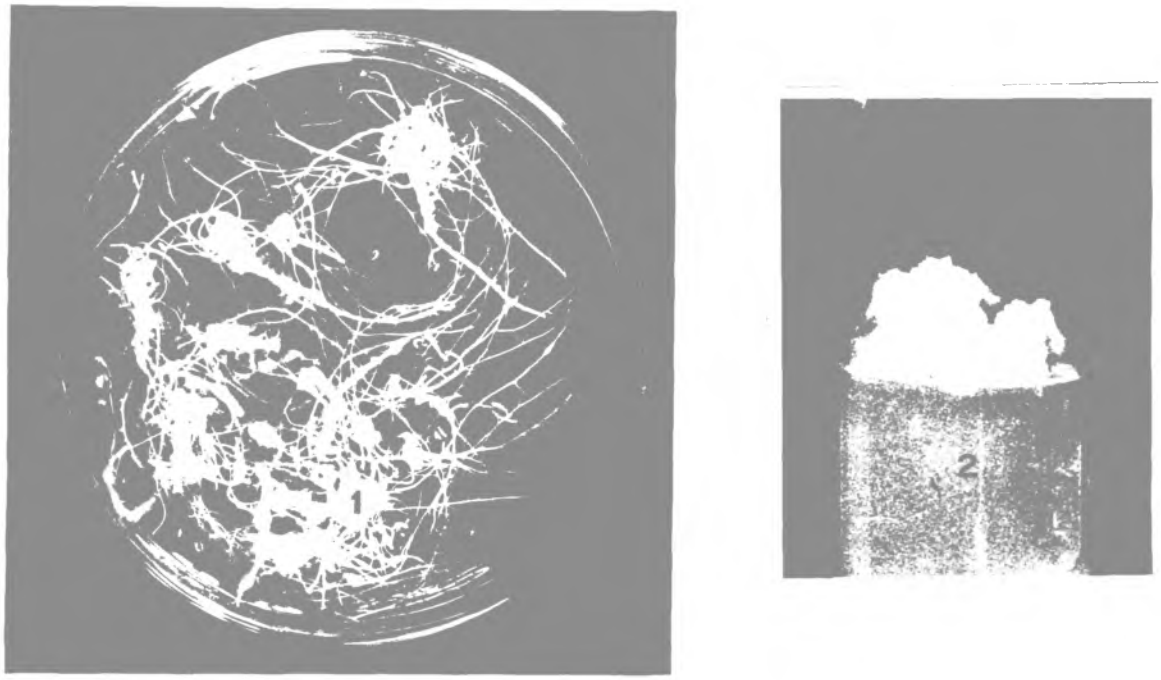


PLATE 6: WHEAT CULTURES GROWN ON BM_2 MEDIUM

(1) SHAKE FLASK (50 DAYS OLD)

(2) AGAR (45 DAYS OLD)

1000 to 1500 mg in 50 days from an initial inoculum of about 200 mg. This growth increase was not much higher than the corresponding growth of these tissues on identical agar media. Sorghum tissue released a red pigment into the liquid medium.

Growing tissues in liquid media is of value for several studies. The utilization of different nutrients in the medium at different periods is more conveniently studied in liquid than in agar media. It is also of value for studying differentiation, since there was a striking difference in differentiation between agar and agitated liquid cultures though the medium was otherwise identical. The very high growth rate in agitated liquid cultures was also of importance in studying the nutritional requirements of these tissues. With slow growing cultures on agar many subcultures over a long period were required to determine whether compounds such as DPU are essential for growth owing to a carry over of nutrient with the inoculum, whereas in shake cultures these experiments could be completed in a much shorter period. This was of particular importance during the earlier part of the work when the growth rate of the cultures was much less than it was after a few years of subculture.

The rest of this chapter deals mainly with the growth of maize tissue in liquid media, its subculture, the effect of a few nutrients on its growth, the utilization of some of the substrates from the medium and the effect of different growth factors on the uptake of phosphorus, nitrogen and sucrose by the tissue. A few studies were also carried out with wheat and sorghum tissues. The basal medium (BM_2P) used for these studies is shown in Table 2D. It differs from the medium used for agar cultures in its phosphate composition. It contained 140 mg/litre of potassium dihydrogen phosphate instead of 70 mg/litre. In earlier

work the lower phosphate concentration was used and though root formation and the rate of growth were striking, the phosphorus in the medium was found to be rapidly used up. To permit studies for longer periods the phosphorus content of the medium was doubled. Any modification in this medium is shown in the respective Tables or Figures. The conditions used for growing these cultures in shake flasks are given in Chapter II.

SECTION 2

Growth curve of maize and wheat

The growth curves of maize and wheat in agitated liquid cultures were then determined. Thirty flasks were used for each tissue and at regular intervals two flasks were removed and the wet and dry weights determined. The inoculum used for the experiments with maize was 200-300 mg of fresh tissue (16 days old) per flask and for wheat the inoculum was 100-150 mg per flask (30 days old). The results are shown in Figures 7 and 8. The volume of the medium in each case was also noted and corrected for evaporation. The culture medium was analysed for sugar, phosphorus etc. described in the next Section.

Maize showed ~~to~~ a lag period of about 5 days, very slow growth for the next 5 or 6 days and rapid growth during the remaining period (Figure 7). The maximum growth rate was a doubling of dry weight in 3 to 3.5 days. The dry weight of the tissue was about 10 per cent of the wet weight during the lag phase and decreased to about 8 per cent on the 27th day.

A similar experiment was also carried out with wheat tissue (Figure 8). Wheat tissue showed a longer lag phase (till the 14th day) during which period the dry weights were 10 per cent of the fresh weight. There was slow growth between the 15th and the 22nd day, rapid growth during the next 10 days and slower growth during the next 20 days.(There were, however, not enough flasks for the period from 32 to 60 days to give significant data, and the data for the 60th day is given only to show that there was no cessation of growth after 30 days.) The water

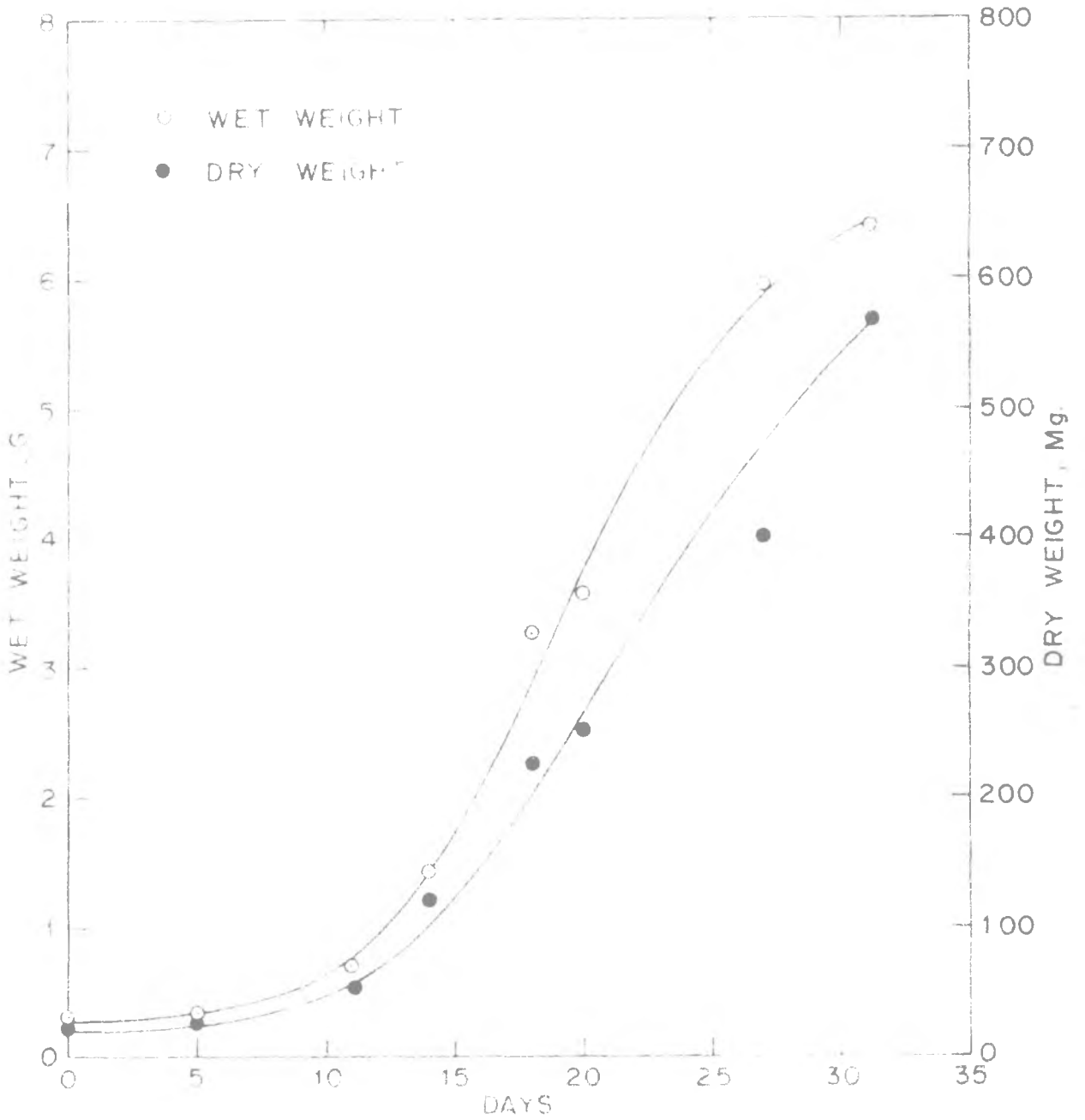


FIG 7

GROWTH CURVE OF MAIZE IN SHAKE FLASKS

MEDIUM: BM2P

INOCULUM 200 – 250 Mg WET

content of the tissue in the final stages was 94 per cent. The growth rate during the period of maximum growth on a dry weight basis was a three-fold increase in 10 days.

As will be seen from the next section the phosphate in the medium was exhausted by about the 20th day in the case of maize and may have limited the growth of this tissue. The dry weights may also not reflect cell division but only increase in cell mass. In view of this the results of Figure 7 require revision with a medium to which nutrients are added periodically to maintain optimum levels and to determine cell numbers.

SECTION 3

Analysis of growth medium

The residual medium obtained at different periods in the previous experiments was used to study the utilization of total organic nitrogen, phosphorus, iron and sugar by the tissues. The results are shown in Tables 39 and 40.

a) Hydrogen ion concentration

The pH of the medium was initially adjusted to 5.8. It will be seen from Tables 39 and 40 that the pH of the medium during 30 to 34 days of growth changed very little and remained between pH 5.0 and pH 6.0 both for maize and wheat.

b) Organic nitrogen

Only organic nitrogen derived from edamin, glycine etc. was estimated. The medium initially contained 0.19 mg nitrogen per ml as determined by the Kjeldahl method. By the 18th day over 50 per cent of the initial nitrogen was utilized by maize tissue. Only a negligible amount of the initial organic nitrogen was taken up by wheat tissue even after 22 days. At the end of 34 days over a tenth of the nitrogen was still present in the maize medium whereas about 40 per cent was present in the wheat medium. The rapid uptake of nitrogen by maize tissue in the first 18 days corresponds to the growth increase of the tissue in its log phase. Till the 22nd day the growth of wheat and utilization of organic N were much less than in the case of maize, but the period of 22 to 31 days was marked by rapid utilization of organic N and rapid growth of the tissue.

TABLE 39

UTILIZATION OF SOME SUBSTRATES BY MAIZE TISSUE IN SHAKE FLASKS

Basal medium: BM_2P

Inoculum: 200–300 mg per flask

Results are the averages for 2 flasks, removed at different times. N, P, Fe and sucrose in the medium were determined after different periods.

Period of incubation	Dry weight	pH	Organic Nitrogen	Total Phosphorus	Iron	"Sucrose"
days	mg/flask		mg/ml	ug/ml	ug/ml	mg/ml
0		5.8	0.190	38.0	6.0	20.0
5	27	5.2	-	29.0	4.8	18.0
11	58	5.8	-	17.0	4.3	17.0
14	124	5.5	-	13.0	4.7	16.0
18	225	5.5	0.084	1.0	-	11.0
20	250	5.3	-	0.3	4.2	11.0
24	205	5.3	-	nil	4.1	-
27	402	5.3	0.065	nil	-	6.5
31	550	5.3	-	nil	3.9	0.2
34	663	5.8	0.026	nil	3.3	nil

TABLE 40

UTILIZATION OF SOME SUBSTRATES BY WHEAT TISSUE IN SHAKE FLASKS

Basal medium: BM_2P

Inoculum: 100–150 mg/flask

Results are the averages for 2 flasks removed at different times. N, P, Fe and "Sucrose" in the medium were determined after different periods.

Period of incubation	Dry weight	pH	Organic nitrogen	Total phosphorus	Iron	"Sucrose"
days	mg/flask		mg/ml	ug/ml	ug/ml	mg/ml
0	10	5.8	0.190	38.0	6.0	20.0
5	11	5.5	-	30.0	4.7	18.4
11	9	5.5	-	-	4.5	-
14	10	5.7	-	29.0	-	17.0
18	24	5.5	-	29.0	-	-
22	40	5.5	0.185	29.0	4.4	17.0
29	54	5.5	-	27.0	-	16.0
31	155	5.5	0.085	24.0	4.5	13.7

The nitrogen content of maize roots grown in shake flasks was found to be 2 mg per gm fresh tissue. The medium originally contained 0.19 mg organic nitrogen per ml or 9.5 mg in the 50 ml used per flask. Of this amount 6.25 mg nitrogen was utilized by the 18th day when the tissue growth was about 3 g or the equivalent of 6 mg nitrogen. Similarly wheat tissue had grown to about 2 g in 31 days, its content of nitrogen was a little over 4 mg, whereas the organic nitrogen taken up from the medium was about 5 mg. These results suggest that most of the nitrogen used by the tissue for growth comes from the organic nitrogen supplements added to the medium.

c) Phosphorus

The basal uninoculated media contained 38 µg of inorganic phosphorus per ml. During the lag period (5 days) nearly 25% of the total phosphorus was taken up by maize tissue and during the next 14 days all the remaining P_i was taken up. With wheat tissue on the other hand there was rapid uptake during the first 5 days whereas less than that amount was taken up during the remaining 26 days. (It should be noted that the inoculum for wheat was only half that used for maize.) On the basis of this experiment subsequent experiments were limited to about 20 days and care was taken that the maximum growth was less than 2.5 to 3.5 g fresh weight which was taken as an index that one of the major nutrients was not limiting growth.

d) Iron

The iron content of the medium was reduced to about half its original amount of 6.0 µg/ml by maize tissue at the end of 32 days whereas only 25 per cent of the initial iron was used by wheat tissue. This may also be due to the fact that the inoculum for maize was only

half that of wheat.

e) "Sucrose"

The total reducing sugar after acid hydrolysis was estimated (which would also include free glucose, fructose etc.) and expressed as "sucrose". The "sucrose" content of the medium for maize showed a gradual decrease with time and was negligible after 31 days. The major amount of sucrose, about 60 per cent, was utilized during the logarithmic period of growth. Wheat tissue on the other hand utilized only 30 per cent of the initial sugar (Tables 39 and 40).

The analytical data from Tables 39 and 40 for maize and wheat indicate a progressive utilization of the medium constituents which were estimated. The phosphorus and sucrose were limiting for maize. The tissue continued to grow slightly even after the depletion of these nutrients due probably to the nutrients inside the cells. It was however observed that if subcultures were made after the period at which all the phosphorus was utilized, there was a longer lag period.

As a result of these experiments the potassium dihydrogen phosphate level was kept at 140 mg per litre instead of 70 mg and sub-cultures from cultures in liquid medium were made before 20 days of growth.

SECTION 4

Effect of nutrients and other factors on growth

The effect of several nutrients, hormones and other compounds, conditioned medium, inoculum size etc. on the growth of maize in agitated liquid cultures (also termed liquid or shake flask cultures) was then determined.

a) Conditioned medium

The effect of "conditioned medium" (or medium in which the cells have been grown for some time) on the growth of maize was determined in view of reports in the literature on the growth promoting effects of conditioned medium (304). In the first experiment 800-900 mg maize tissue was inoculated into 2 shake flasks each containing 100 ml medium. After 11 days a weighed amount of tissue from each flask and 50 ml of conditioned medium were transferred to flasks containing 50 ml of fresh medium. The flasks were incubated on the shaker for 8 days at the end of which period a similar procedure was adopted transferring 50 ml of the "conditioned medium" with weighed amounts of the tissue as inoculum to fresh flasks. This was repeated over 6 subcultures and the growth noted each time (Table 41). The average growth increase (as indicated by the ratio of the final fresh weight to the fresh weight of the inoculum) doubled in the second subculture from 2 to 4. The final weight was only 1.5 g from about 0.8 g of inoculum in 11 days in the first experiment without conditioned medium, whereas in the next subculture which contained "conditioned medium", the final weight was 3.0 g from nearly the same inoculum in only 8 days. Subsequent transfers showed no further increase in growth rate. Further studies varying both the ratio of "conditioned" to fresh medium and inoculum

TABLE 41

EFFECT OF CONDITIONED MEDIUM ON MAIZE TISSUE IN SHAKE FLASKS

100 ml basal medium BM_2P was added to each of the two 500-ml flasks and inoculated with maize tissue. At the end of 8 to 10 days a weighed amount of tissue was removed together with 50 ml of the "conditioned" medium and transferred to 500 ml Erlenmeyer flasks containing 50 ml of fresh medium. Subcultures and growth measurements were made every 8 to 11 days. This experiment was repeated over 6 subcultures.

	Subculture number	Inoculum wet weight	Period of incubation	Weight		Ratio of Final weight / Inoculum weight
				Wet	Dry	
		mg/flask	days	g/flask		
1	I	800- 900	11	1.5	-	2.0
2	II	700- 800	8	3.0	-	4.0
3	III	900-1000	9	3.0	0.20	3.1
4	IV	900-1000	10	3.75	-	4.0
5	V	1000-1100	9	4.5	0.27	4.2
6	VI	1000-1100	9	4.8	0.28	4.5

sizes etc. and measuring the growth by cell counts would be necessary before drawing any conclusions from this experiment. The effect of conditioned medium in reducing the lag phase or increasing the initial growth of the culture is however significant.

b) Effect of inoculum size

The following experiment was carried out to ascertain whether any relation existed between the size of inoculum and the growth rate. Inocula weights less than 100 mg were not used because of preliminary studies which showed very poor growth in shake flasks. There was an increase in growth rate (final weight/initial weight) as the inoculum was increased from 100 to 400 mg (Table 42). However in this experiment the final growth was comparatively poor (possibly due to using inoculum grown for more than 20 days). The inoculum size in all further experiments was kept at 200-300 mg/flask.

c) Effect of cytokinins

Kinetin concentration: The effect of different concentrations of kinetin on the growth of maize tissue in shake flasks is shown in Figure 9. The increase in the fresh weight was not significant at any of the concentrations tested though the dry weights of maize tissue with kinetin were significantly higher at 0.01 ppm kinetin relative to the controls.

Zeatin concentration: In agar media maize tissue in the first subculture was stimulated by zeatin this stimulation being reduced in the second and third subculture. In shake flasks when zeatin was added at the very low concentration of 0.025 ug per litre, the fresh and dry weights were not different from those of the controls without zeatin. At concentrations of 0.05 ug/litre or higher the growth was markedly inhibited (Figure 10).

TABLE 42

EFFECT OF DIFFERENT SIZES OF INOCULUM ON GROWTH OF MAIZE TISSUE IN SHAKE FLASKS

Basal medium: BM_2P

Period of incubations (days): 20

	Inoculum wet weight	Weight		Final wet weight / Inoculum weight
		Wet	Dry	
	mg/flask	mg/flask		
1	100	350	29	3.5
2	200	654	52	3.2
3	300	1330	115	4.4
4	400	2074	154	5.0
5	500	2449	192	4.8

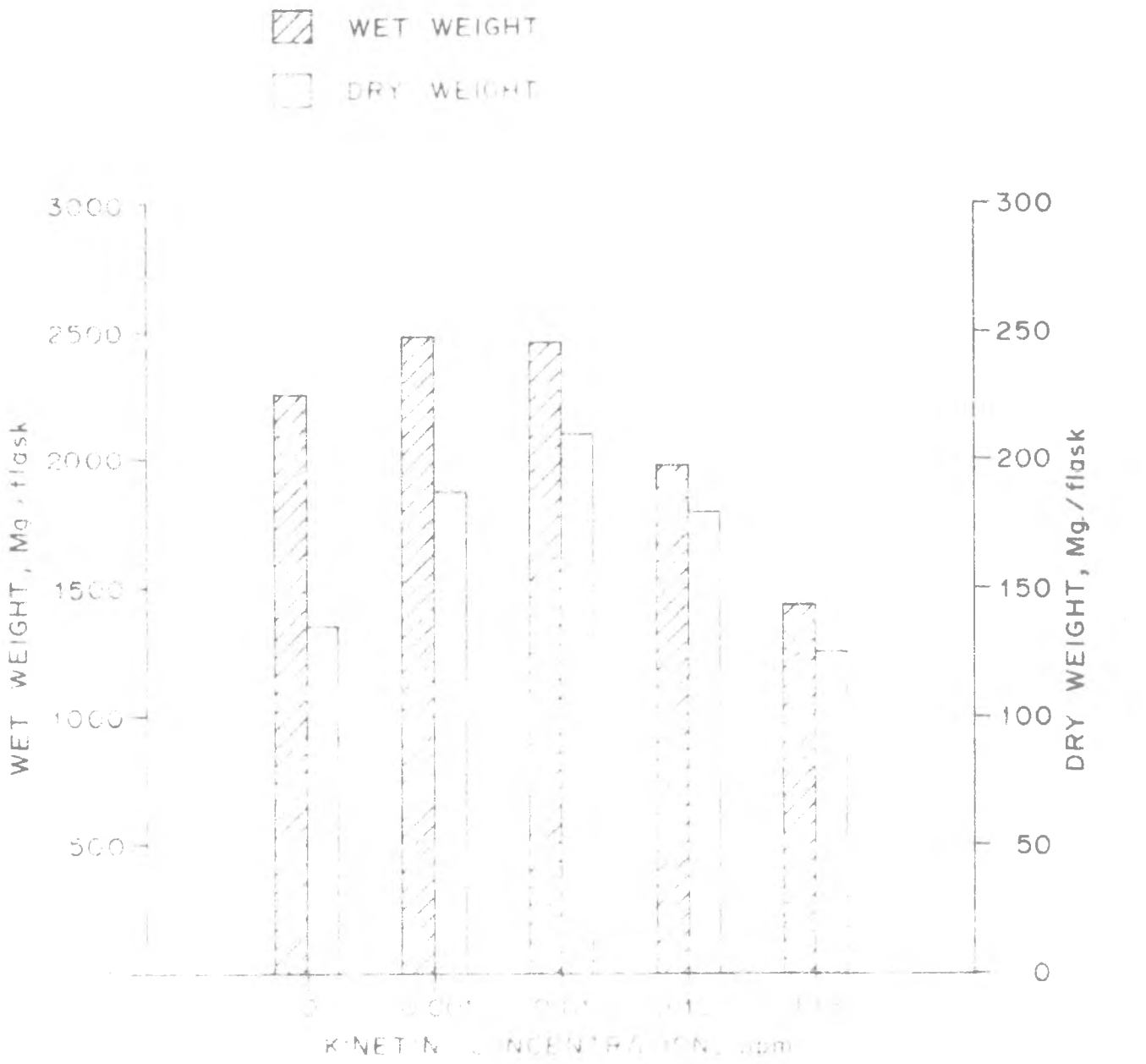
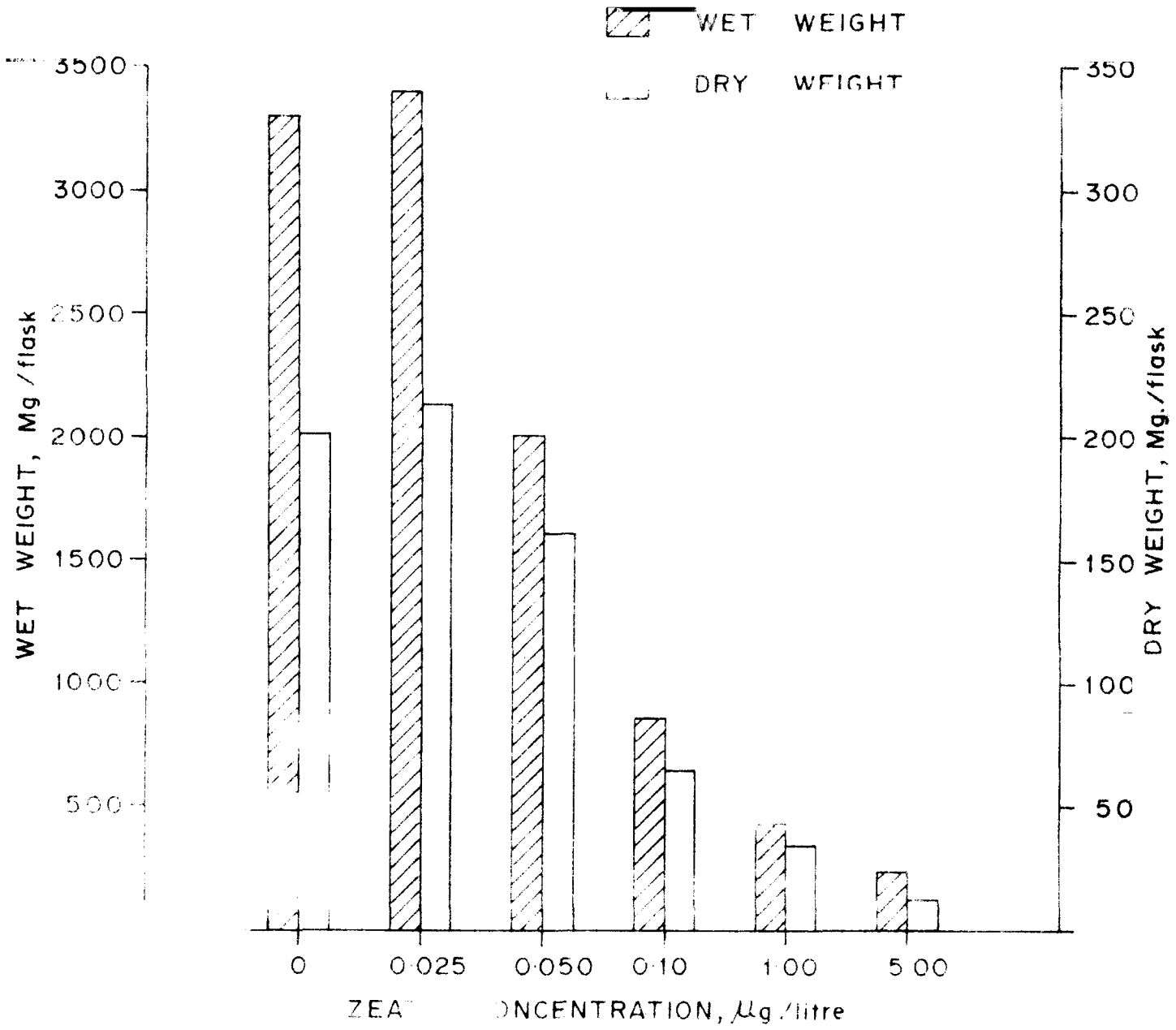


FIG 9

EFFECT OF KINETIN CONCENTRATION ON GROWTH OF MAIZE TISSUE IN SHAKE FLASKS

MEDIUM BM2P (NAA AT 1 ppm) (KINETIN ADDED AT DIFFERENT CONCENTRATIONS)
INOCULUM 200 – 250 Mg WET
PERIOD 20 DAYS



EFFECT OF ZEATIN CONCENTRATION ON GROWTH OF MAIZE TISSUE IN SHAKE FLASKS

MEDIUM: BM 2P (ZEATIN ADDED AT DIFFERENT CONCENTRATIONS)
INOCULUM: 200–250 Mg WET
PERIOD: 20 DAYS

The effect of zeatin was also tested at 2 concentrations 0.025 and 0.050 ug/litre on the growth of maize tissues in shake flasks on repeated subculture. There was no reduction in growth rate even after 27 subcultures at either of the concentrations of zeatin tested. In fact the growth rate was markedly higher in subcultures maintained in the presence of zeatin (Table 46, Media 9 and 10). This may be due ~~either~~ to selection of more rapidly growing strains in the presence of zeatin. However, there is no requirement for kinetin or zeatin for these cultures in shake flasks and the effect of zeatin is noticeable only in long term experiments.

Diphenylurea concentration

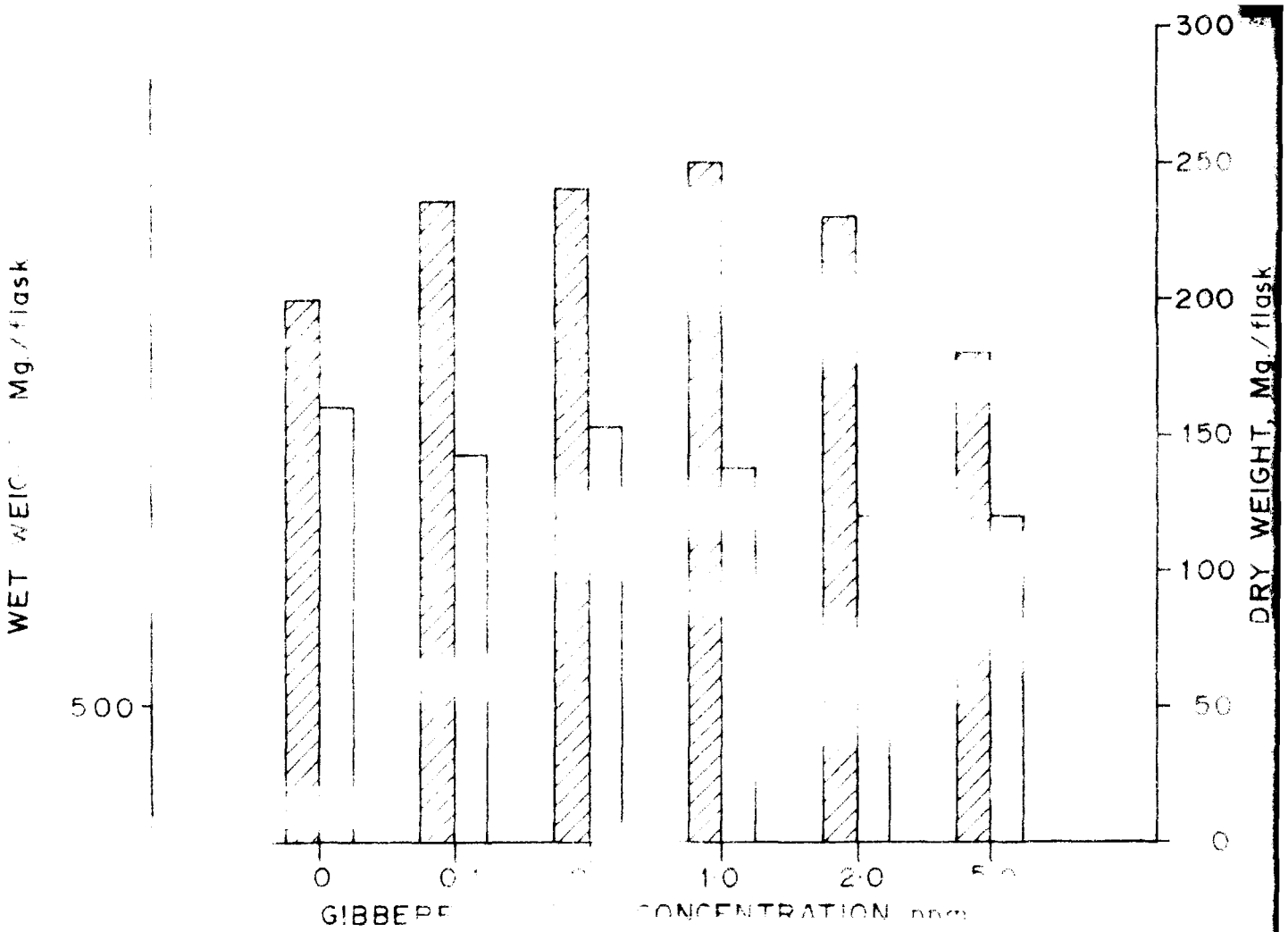
As seen from Figure 11 the optimum concentration of DPU for the growth of maize tissue in shake flasks was 0.1 ppm. At this concentration the increase in fresh weight as compared to the control without DPU was about 70 per cent and the increase in dry weight about 30 per cent. At 0.5 or 1.0 ppm DPU the growth was approximately the same as that of the controls without DPU. More than 50 per cent inhibition was observed at a concentration of 5 ppm DPU as compared to the controls without this substance.

d) Effect of gibberellic acid (GA_3)

Gibberellic acid concentration: Gibberellic acid (GA_3) does not affect to any noticeable degree the fresh or dry weights of maize tissue in the concentration range tested (0.1 to 5.0 ppm) when compared with the controls without gibberellic acid (Figure 12).

e) Effect of growth retardants and abscisic acid

Two growth retardants CCC and AMO-1618 were tested at two concentrations on the growth of maize tissue in shake flasks. With CCC



EFFECT OF GIBBEREL ATION ON GROWTH

MAIZ SSUE HAKE FLASK

MEDIUM: BM ADDED FERENT CONCENTRATIONS)

INOCULUM: 200 Mg. WE

PE 20 DAYS

TABLE 43

EFFECT OF GROWTH RETARDANTS AND ABSCISIC ACID ON MAIZE TISSUE IN SHAKE FLASKS

Basal medium: BM_2P

Period of incubation: 20 days

The growth retardants and abscisic acid were added to the medium at 2 concentrations (0.1 and 1.0 ppm).

	Retardant added with concentration		Weight	
			Wet	Dry
		ppm	mg/flask	
1		-	3811	257
2	CCC	(0.1)	472	43
3	CCC	(1.0)	650	57
4	AMO-1618	(0.1)	2710	207
5	AMO-1618	(1.0)	529	43
6	Abscisic acid	(0.1)	573	43
7	Abscisic acid	(1.0)	258	21

maize tissue growth was markedly inhibited even at 0.1 ppm (Table 43). With AMO-1618 there was a 20 per cent inhibition of growth at 0.1 ppm and very little growth at 1.0 ppm. Abscisic acid also inhibited the growth of maize tissue markedly even at the lower concentration tested (0.1 ppm).

Effect of abscisic acid using different inocula:

When the effect of abscisic acid was tested at 0.1 ppm concentration under shake flask conditions and using a liquid grown maize root inoculum, the inhibition observed was almost total (Table 43) as compared to only about 50 per cent observed on solid agar medium (Table 33, Chapter III). These findings would suggest a selective inhibition of root growth by abscisic acid while the callus is not affected so markedly. To test this possibility more rigorously, maize tissue from agar cultures consisting of callus and root and from liquid media consisting solely of roots were inoculated separately into liquid media with (0.1 ppm) and without abscisic acid. The inoculum used in each case was 200-250 mg per flask and the flasks were incubated for 20 days. The results indicate (Table 44) a complete inhibition of the growth of maize tissue from shake flask inocula (roots), but only about 50 per cent inhibition of the growth of tissue from agar inocula (roots plus callus).

f) Effect of auxin

Naphthalene acetic acid concentrations: From Figure 13 it will be seen that maize shows maximum growth at 5 ppm NAA. At higher and lower concentrations there was a marked fall in growth, with virtually

TABLE 44

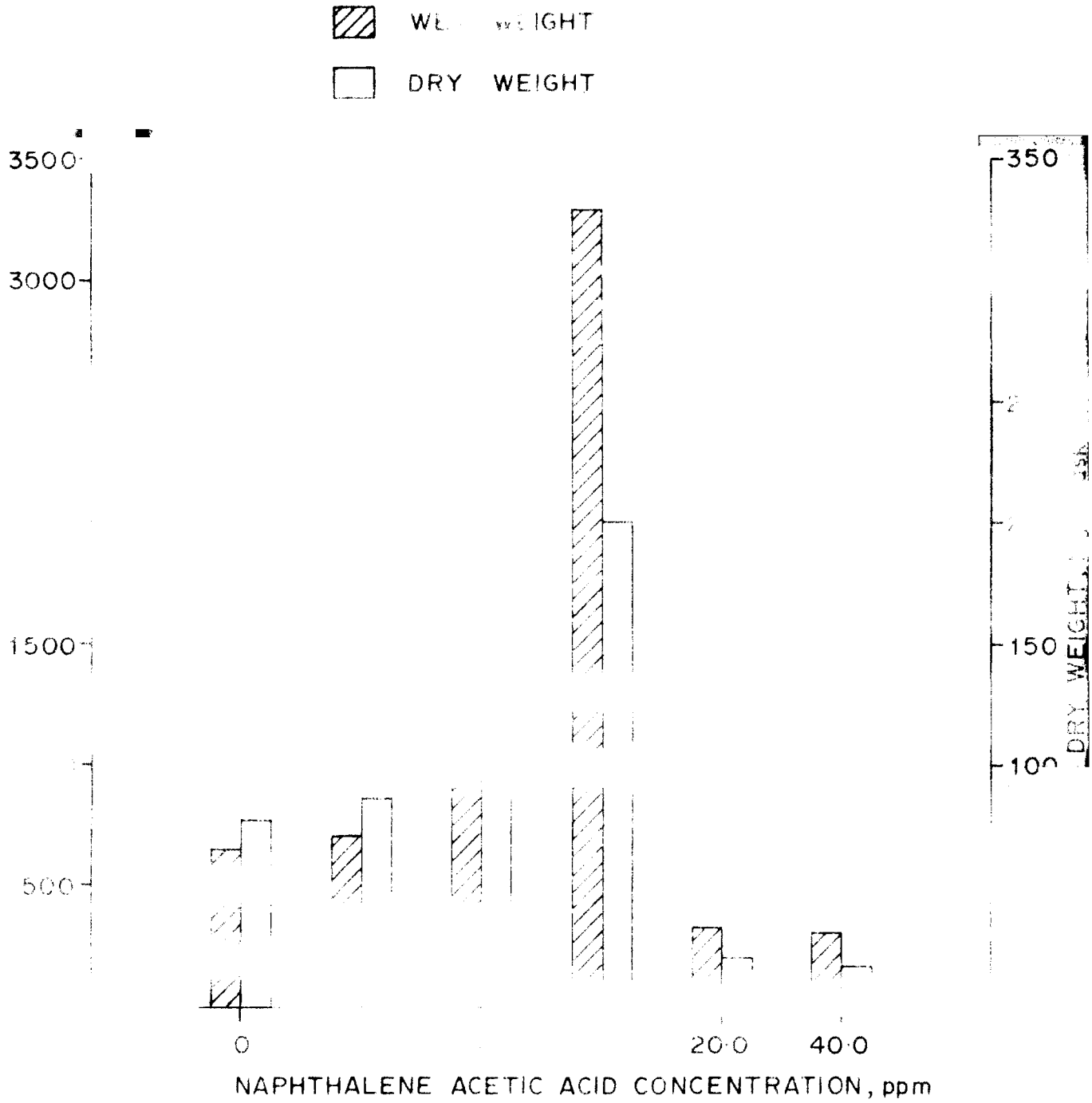
EFFECT OF ABSCISIC ACID ON AGAR GROWN OR SHAKE FLASK INOCULA OF MAIZE TISSUE IN SHAKE FLASKS

Basal medium: BM_2P

Inoculum weight: 200–250 mg/flask

Period of incubation: 20 days

	Source of inoculum	Abscisic acid added	Weight	
			Wet	Dry
		ppm	mg/flask	
1	Shake flask	-	3945	207
2	Shake flask	Abscisic acid (0.1)	352	27
3	Agar culture	-	2315	263
4	Agar culture	Abscisic acid (0.1)	1620	148



EFFECT ENTRATION ON GROWTH OF

MAIZE SHAKE FLASKS

MEDIUM: BM2P (NAA ADDED AT DIFFERENT CONCENTRATIONS)

INOCULUM: 200–250 Mg. WET

no growth at 20 ppm or more of NAA. The dry weights of the tissue were significantly high when grown without any auxin or at a low auxin concentration (0.1 ppm).

g) Effect of vitamins

Effect of single omission of White's 'B' supplements: Thiamine, pyridoxine, niacin and glycine (White's 'B') were added at the high concentrations specified in Table 2C, in all the studies on the growth of maize, wheat, rice and sorghum. To test whether any of these substances were actually essential for growth would be very difficult to confirm in agar media where carry over of nutrients and slow growth rate would offer difficulties. The increased growth rate of maize tissue in shake flasks would reduce these problems and would confirm the effect of a particular nutrient in a shorter time. In this experiment (Table 45) the effect of the individual substances, thiamine, pyridoxine, niacin and glycine were tested by omission of any one of these supplements at a time in each experiment. The results indicate that glycine, pyridoxine and thiamine are needed for optimum growth of maize tissue in shake flasks, since there was a growth inhibition of about 50 per cent in their absence. The tissues grew as much as the controls containing all these supplements even when nicotinic acid was omitted from the medium indicating that maize tissue did not require this vitamin. Agar grown maize cultures also showed a requirement for thiamine but not for pyridoxine. It should also be noted that the net increase in weight in shake flasks was not so great as to reduce carry over of nutrients very greatly. The marked lowering of growth in the absence of B_1, B_6 and glycine may be due to a greater requirement of these for root growth compared to callus on solid media or different degrees of accumulation of these compounds under the two different conditions of growth.

TABLE 45

EFFECT OF SINGLE OMISSION OF THIAMINE, PYRIDOXINE, NICOTINIC ACID AND GLYCINE ON MAIZE TISSUES IN SHAKE FLASKS

Basal medium: BM_2P

Changes: Edamin omitted. Tyrosine added at 2 ppm. One vitamin or glycine was omitted from control medium in each experiment except medium 2. (Concentrations of these chemicals given in Table 2C.)

Period: 20 days

Inoculum (mg): 200-250

	Vitamins or glycine omitted	Weight	
		Wet	Dry
		mg/flask	
1	Control medium	2738	186
2	All vitamins and glycine	388	33
3	Glycine	1317	126
4	Nicotinic acid	2604	201
5	Pyridoxine hydrochloride	1179	81
6	Thiamine hydrochloride	1591	100

Inositol concentrations:- Inositol was added to all media at 100 ppm. The effect of this substance at different concentrations on the growth of maize tissue in shake flasks was determined at different levels of this compound. In the absence of any inositol the growth of the tissue was comparatively poor, there being a gradual increase in the growth of the tissue with increase in concentration of inositol upto 100 ppm and an inhibition of growth at 500 ppm (Figure 14). Agar grown maize cultures did not show a requirement for inositol at any of the concentrations tested.

h) Edamin concentrations

Different edamin concentrations were tested in shake flasks for their effect on maize. In the absence of edamin the growth of the tissue was very poor. A progressive increase in growth was observed as the concentration of edamin was raised from 0.001 to 0.1 per cent, 0.1 per cent being the optimum. At a level of 1.0 per cent the growth of the tissue was inhibited (Figure 15).

i) Effect of subculture on maize in shake flasks

In agar media maize tissue was routinely maintained by subculture every 45 days on media of 20 different compositions. After it was found that tissue from agar grown cultures grew vigorously in liquid media, a number of different media were prepared in which maize tissue was grown and routinely subcultured every 18 to 20 days to study the effect of prolonged subculture on the growth of the tissue under these conditions (Table 46). (Experiments 1-4 are at low P_i and experiments 5-10 at twice the P_i concentrations. Growth in the two sets cannot be compared.)

The tissues were subcultured in some cases for over 3 years. The results indicated that for long term growth of maize tissue in shake flasks:-

1) DPU, zeatin and glycine were not essential for growth.

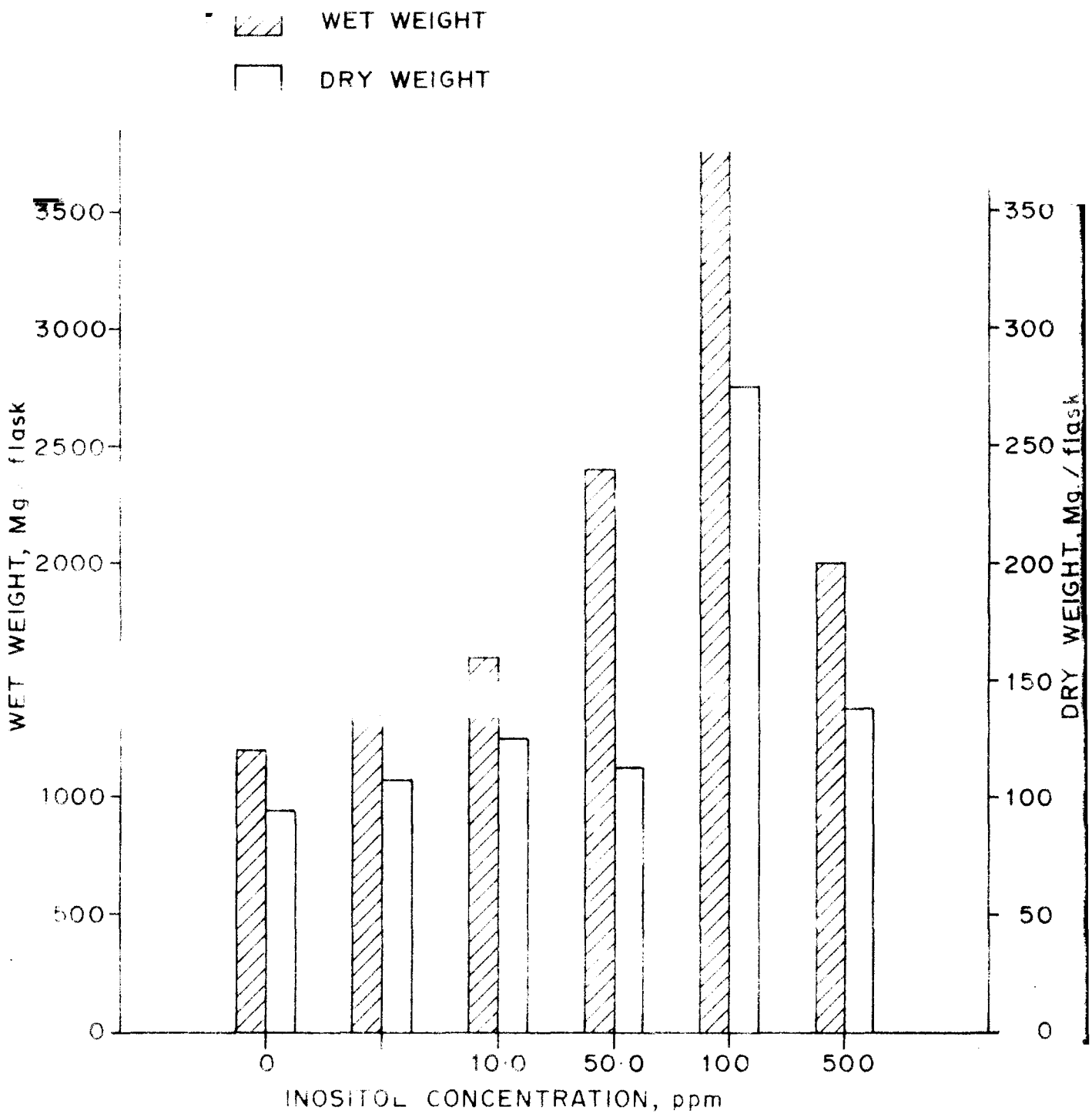


FIG. 14.

EFFECT OF INOSITOL CONCENTRATION ON GROWTH OF MAIZE TISSUE IN SHAKE FLASKS

MEDIUM: BM2P (INOSITOL ADDED AT DIFFERENT CONCENTRATIONS)

INOCULUM: 200 - 250 Mg. WET

PERIOD: 15 DAYS

TABLE 46

DIFFERENT MEDIA ON WHICH MAIZE CULTURES WERE MAINTAINED (SHAKE FLASKS)

Mineral salts: (Macro and micro) Smith's (Tables 2A & 2B). In some experiments KH_2PO_4 was added at 140 mg instead of 70 mg/litre (Media 5 to 10).

Vitamins: Table 2C. Glycine omitted in some experiments (Media 1 and 2).

Other supplements: As in Table.

Inoculum: 200-250 mg wet.

	Phosphate	Glycine	Carbohydrate	DPU	Zeatin	NAA	Edamin	Inositol	Wet weight at last passage	Number of passages	Period
	mg/l	30 ppm	2%	ppm	ug/litre	ppm	1000 ppm	100 ppm	mg/flask		months
1	70	-	Sucrose	1.0	-	1.0	+	+	1377	60	36
2	70	-	Glucose	1.0	-	1.0	+	+	1051	60	36
3	70	+	Sucrose	1.0	-	1.0	+	+	1512	57	35
4	70	+	Sucrose	-	-	1.0	+	+	1500	54	33
5	140	+	Sucrose	-	-	5.0	+	+	1736	54	33
6	140	+	Sucrose	1.0	-	5.0	+	+	1433	54	33
7	140	+	Sucrose	0.1	-	1.0	+	+	989	54	33
8	140	+	Sucrose	0.1	-	5.0	+	+	4300	54	33
9	140	+	Sucrose	-	0.025	5.0	+	+	5087	32	19
10	140	+	Sucrose	-	0.050	5.0	+	+	4514	32	19

2) Either glucose or sucrose could be used as a carbohydrate source.

3) Diphenylurea was not essential but low concentrations of this cytokinin greatly increased growth.

4) α- naphthalene acetic acid was more stimulatory for growth at 5 ppm than at 1 ppm.

5) Zeatin at 0.025 and 0.050 ug/litre gave better growth.

As discussed earlier these results must be regarded as tentative unless it is possible to clone these cultures. It is possible that the original culture is a mixture of different strains and during prolonged culture, strains which can survive on glucose or without glycine or which respond better to zeatin or DPU may have been selected.

j) Wheat and sorghum in shake flasks

Wheat and sorghum tissues were also maintained as roots on medium 6 of Table 46 by repeated subculture. Wheat roots grew to about 2000 to 2500 mg within 30 days, whereas sorghum grew to 1000-1500 mg in 50 days from an initial inoculum of about 200 mg. Wheat has undergone over 25 passages with subcultures every 30 days and sorghum 10 passages the subculture period being 50 days. These tissues, like maize tissue in shake flasks, grew as a mass of roots.

These experiments show that root cultures of maize, wheat and sorghum can be maintained *in vitro* on prolonged subculture in shake flasks. This is the first report, as far as the author is aware, of the long term growth of plant roots in vigorously agitated liquid cultures and also the first successful culture of maize and sorghum roots which are viable *in vitro*.

S E C T I O N 5

Effect of growth factors on uptake of certain ions

In this set of experiments the interaction of different cytokinins with auxin was studied to determine whether the uptake of sugar, phosphorus and organic nitrogen was retarded or enhanced in their presence. In the first experiment White's modified macro and micro mineral salts and vitamins were used at the levels shown in Tables 2A, 2B and 2C. However 88 mg $NaH_2PO_4.2H_2O$ was added instead of 22 mg/litre as given in Table 2A. To this medium supplements of inositol and edamin were added together with the respective cytokinins under test. After 18 days the tissue was weighed and the sugar, phosphorus and organic nitrogen content determined in the spent medium to see whether the utilization of these three substrates was affected by cytokinins. The results of the experiments in this Section were confirmed by repetition under identical conditions (Table 47).

The concentrations of the kinins were such as to inhibit growth and the concentrations of auxin were not optimum. An increased uptake of sucrose, phosphorus and nitrogen was observed in the presence of kinetin alone or coupled with zeatin. This effect of kinetin was partly reversed by DPU. High NAA and kinetin showed the same uptake as the control. These experiments are to be regarded as preliminary experiments to determine whether these compounds had any effect on the uptake of these nutrients. It is of interest that kinetin enhances uptake of phosphorus and nitrogen in spite of inhibition of growth. Further experiments preferably over a shorter period and a wider concentration range of these compounds would be of interest.

TABLE 47

GROWTH AND UTILIZATION OF PHOSPHORUS, SUCROSE AND ORGANIC NITROGEN FROM THE MEDIUM IN THE PRESENCE OF DIFFERENT GROWTH FACTORS

The medium used in this experiment was that of White but contained 88 mg $NaH_2PO_4.2H_2O$ instead of 22 mg/litre. The other macro and micro salts, vitamins and glycine were added at the concentrations given in Tables 2A, 2B and 2C. The concentrations at which the growth factors were added are given in column 2.

The initial values for sucrose were 21.0 mg; for phosphorus 20 ug; and for organic nitrogen 0.192 mg per ml medium.

Inoculum: 180-200 mg wet equal to 15-17 mg dry.
Period: 18 days.

	Growth factor added	Wet weight	Dry weight	Sucrose utilized	Phosphorus utilized	Organic nitrogen utilized
	ppm	mg/flask		mg/ml	ug/ml	mg/ml
1	NAA (1)	2100	200	6	15.0	0.126
2	NAA (1) + Kinetin (1)	1610	144	9	19.8	0.153
3	NAA (1) + Kinetin (1) + Zeatin (0.001)	945	98	8	19.9	0.143
4	NAA (1) + Kinetin (1) + DPU (1)	670	61	5	12.0	0.104
5	NAA (10) + Kinetin (1)	1096	95	6	16.0	0.129

TABLE 48

EFFECT OF DIFFERENT NAA AND PHOSPHATE CONCENTRATIONS ON GROWTH OF MAIZE AND ON UTILIZATION OF PHOSPHORUS FROM THE MEDIUM

The medium was BM_2 having the phosphate and NAA concentrations as given in the Table.

Wet weight of inoculum: 150-200 mg/flask.

Period of incubation: 18 days.

	Amount of KH_2PO_4 added	NAA concentration	Wet weight	Estimated initial phosphorus	Phosphorus utilized
	mg/l	mg/l	mg/flask	µg/ml	ug/ml
1	0	0.1	297	0	0
2	140	0.1	380	38	27
3	700	0.1	564	202	57
4	0	1.0	517	0	0
5	140	1.0	704	38	28
6	700	1.0	755	202	70
7	0	5.0	159	0	0
8	140	5.0	2266	38	31
9	700	5.0	2206	202	84

TABLE 49

EFFECT OF DIFFERENT NAA AND PHOSPHATE CONCENTRATIONS ON GROWTH OF WHEAT AND ON THE UTILIZATION OF PHOSPHORUS FROM THE MEDIUM

Medium was BM_2 having the phosphate and NAA concentrations as given in the Table.
Wet weight of inoculum 150-175 mg/flask.
Period of incubation 30 days

	Amount of KH_2PO_4 added	NAA concentration	Weight		Estimated initial phosphorus	Phosphorus utilized
			Wet	Dry		
	mg/l	mg/l	mg/flask		ug/ml	ug/ml
1	70	0.1	169	9	22	9
2	350	0.1	433	42	101	31
3	700	0.1	221	21	207	76
4	70	5.0	1244	109	22	15
5	350	5.0	648	61	101	35
6	700	5.0	622	52	207	78

SECTION 6

Discussion

Suspension cultures have been obtained from a number of cultured plant tissues by techniques involving the transfer of tissue fragments or callus from agar cultures to agitated liquid media (51,138,196,279). Examination of such suspensions in all cases revealed the presence not only of free cells, but also of cell aggregates ranging within each culture from groups of 2 to 10 cells upto groups of several hundred cells. Carew and Staba (56) classified liquid cultures of plants grown on mechanical shakers into 3 categories; (1) uniformly dispersed cells as reported by Lamport (138), Lin and Staba (149), Nickell (196); (2) the more common cell aggregates described by Nickell and Tulecke (204). Such tissue aggregates transferred to media having higher levels of growth regulating agents like YE and 2,4-D gave a free cell suspension (21,67,138,320); (3) organized structures such as roots or embryoids and plantlets (98, 269,284).

Liquid cultures are potentially more useful when one wishes to study the metabolism of the plant cells during growth, the medium being more readily sampled or replenished. Liquid cultures also enable one to obtain large amounts of tissue by growing them in carboys or fermentors (177,204,328,330,340). Such large amounts of tissue are useful for the production of useful metabolites (53,266,269). Suspension cultures are also useful in obtaining dissociated cells for studies on the growth of single cells etc.

However when maize, wheat or sorghum tissues grown on agar media were transferred to agitated liquid media, the tissue did not separate

into cells or grow as aggregates, but developed into an organized structure consisting of a fibrous mass of roots which could be sub-cultured at regular intervals. It should be emphasized that these results are unique in several ways: (1) The growth of plant organs such as roots when shaken at such high rates as 180 rpm has never been reported before. A large number of dicotyledonous plant cell cultures have been grown in shake flasks in this laboratory and in others, but the rapid continuous growth of organized tissues such as roots has not been reported hitherto under conditions of such vigorous agitation. Maize, wheat and sorghum roots not only do not disintegrate when shaken vigorously but grow very rapidly as roots. Earlier work on plant roots has been restricted mainly to stationary liquid cultures. (2) The main difference between the agar cultures and the liquid cultures in addition to the absence of agar was vigorous agitation. There was no difference in the composition of the medium. (Other experiments, which will not be described here, on the effect of light, phosphate concentration, different O_2 or CO_2 partial pressures showed that they had no effect on the amount of roots relative to callus in solid or liquid cultures with maize.) Earlier work on differentiation, especially of roots, has been concerned with the effect of auxin, kinetin, gibberellin, adenine etc. This is the first report on rapid formation and growth of roots in liquid cultures from tissue which on agar with the same medium grows as a mixture of callus and roots (maize) or mainly callus (wheat). Hormones do affect the relative amounts of callus and roots in these cultures as will be described later, but the effect of agitation in liquid is far more striking since it gives exclusively roots. This observation permits several studies which would otherwise be difficult (eg. the selective effect of abscisic acid on root growth compared to callus growth).

(3) This differentiation is reversible. Transfer of roots from liquid cultures to agar gives once again a mixture of callus and roots with maize and wheat. Such a reversible differentiation by a very simple cultural technique has not been reported earlier, as far as the author is aware. Nucleic acid metabolism and enzyme and protein synthesis can be readily studied in such a system where no other effects due to change in concentration of auxin, kinetin etc. are superimposed. (4) These cultures of maize, wheat and sorghum are root cultures capable of rapid growth and viable indefinitely on subculture. Wheat root cultures have been reported earlier (70,323). Maize and sorghum roots have never been grown in continuous culture previously and these are the first viable root cultures of these two monocotyledonous plants.

Growth curve of maize and wheat

Lin and Staba (149) grew peppermint and spearmint tissues in submerged culture and studied their rate of growth over a 4 week period. Most growth occurred between the 5th and the 14th day after which period the growth was very slow. Lamport (138) studied the growth curve of sycamore cells by aseptic sampling and noting the packed cell volumes of 10 ml samples. He found a steady rise in growth from the first to the 10th day of these cell suspensions. Nickell and Tulecke (202) compared the growth rates of samples of carboy cultures to tissue grown in Erlenmeyer flasks in agitated conditions and found accelerated rate of growth in carboy cultures in the first two weeks as compared to the growth in shake flasks.

The growth pattern for maize and wheat tissue in shake flasks (Figure 7) is basically similar and shows no unique features. The inoculum in these experiments was from agar. If it had been taken from

shake flasks in the log phase, the lag period might have been reduced and linear increases in growth might have been observed from the initial stages (Table 41). The addition of conditioned medium along with the inoculum may also be of value in such studies. The concentrations of nutrients, especially of phosphate and sucrose, requires adjustments to optimum values to obtain more rapid growth.

Maize showed a lag period of about 5 days compared to a lag of 14 days by wheat tissue. This was followed by the exponential phase which continued till about the 32 day. The water content of both maize and wheat was lowest during the lag and early log phases, the amount increasing at the late log and stationary phases (Figures 7 & 8). During the most active growth period the doubling time as judged by weight was 3 to 3.5 days.

Analysis of the medium

Though there are many reports on growth of liquid suspension cultures, reports on the rates of utilization of nutrients in the medium are relatively rare. Such time-course studies on the rates of tissue growth and utilization of medium constituents could lead to improved media and to better growth.

pH

Lin and Staba (149) in studies with submerged cultures of peppermint observed only slight fluctuations in pH (5.5 - 6.5) over a 4-week period. Maize and wheat cultures maintained a constant pH in the medium, though the phosphate which is the main buffering constituent in the medium was virtually exhausted in the case of maize.

Organic nitrogen

As stated earlier the tissue preferentially utilizes organic nitrogen in the medium since the N content of the tissue corresponds to the organic N utilized on the 18th day. The results with wheat are similar. A direct estimation of the nitrate content of the medium was however not made at different periods of growth.

Gamborg and Eveleigh (79) added a nitrogen source, N-Z amine type A, at 2 ppm in the medium for culturing wheat and barley. Nickell and Maretzki (200) found in experiments using casein hydrolyzate as a supplement in place of yeast extract that the growth of sugarcane cell suspensions was very poor. Using a mixture of amino acids they could obtain on a synthetic medium only 60 per cent of the growth on White's basal medium plus yeast extract plus arginine. Tiwari and Arya (315) studied the effect of 4 different types of casein hydrolyzate on the growth of normal and diseased Pennisetum tissues and found that Oxoid hydrolyzed casein hydrolyzate was the best and pancreatic digest of casein hydrolyzate (type A of Sheffield Company) was the least effective for growth of these tissues. For maize tissue the optimum concentration of edamin is 0.1 per cent (Figure 15).

Phosphorus

Bellamy and Bieleski (17) studied the growth pattern of tobacco cells in suspension culture and noticed that the end of the exponential phase and the start of the early stationary phase were accompanied by a marked fall in the concentration of phosphate in the culture medium. The rates of accumulation of phosphorus in the tissue were highest at the early to mid stationary phase of growth.

With maize tissue also there was a marked uptake of phosphorus during the exponential phase, a marked drop being observed during the latter part of the log phase due partially to limited availability of phosphorus. With wheat tissue, on the other hand, there was rapid uptake during the first 5 days, whereas a negligible amount was utilized during the remaining growth period.

Iron

Iron was not limiting for the growth of maize and wheat when the initial amount was 6 ug/ml.

"Sucrose"

Wang and Staba (340) studied the utilization of sucrose from dual carboy cultures of spearmint tissues grown under aeration. They found that by the third day sucrose was completely hydrolyzed to dextrose and fructose both of which were absent in the culture medium after 8 days. Simpkins et al (260) obtained data from the growth of Acer pseudoplatanus cells in submerged culture to show that all the sucrose from the medium was depleted after 20–25 days of incubation, the medium initially containing 2 per cent sucrose.

The utilization of sugars from the medium expressed in terms of "sucrose" in the present experiments indicated a complete uptake from the medium by maize tissues by the 34th day, whereas wheat tissues had utilized only 30 per cent of the original sucrose. No attempt was made to study the hydrolysis of the sucrose (Tables 39 and 40).

Conditioning of medium

Muir, Hildebrandt and Riker (188) showed that isolated single cells could be made to divide if cultured on an actively dividing

callus culture separated only by a filter paper, the actively dividing callus culture acting as a nurse tissue to the isolated cell. Similarly when as a consequence of tissue growth a medium develops enhanced growth promoting activity which can change the lag phase of growth to the exponential phase it is known as a "conditioning" of the medium. Stuart and Street (304) described techniques for "conditioning" a culture medium by separation from a dense cell suspension either by a sintered glass filter or a dialysis membrane. To obtain a conditioned medium of high activity by which the lag period was shortened and growth of low density cell cultures occurred in new medium, an appropriate volume ratio of culture medium to conditioning cell suspension and a limiting of the culture period were found necessary. Staling of medium and accumulation of products which inhibit growth have also been reported with plant cultures (304) and the relative amounts of activators and inhibitors of growth probably vary with the time and other experimental conditions.

In the case of maize tissue a reduction inthe lag period was observed when a 50:50 ratio of conditioned to fresh medium was inoculated with 8 to 10 day old tissue grown in the same medium used for the conditioning (Table 41). However since the inoculum weight in these experiments was very high and the volume of medium 100 ml instead of 50 ml, the reduction in the lag phase could also have been due to these factors since Syono and Furuya (307) observed that lag of growth was longer with small than with large inocula of tobacco callus in liquid culture and that the different responses between small and large inocula were dependent on the ratio of inoculum to culture medium. The increase in growth rate (Table 41) could also be due to the fact that the first inoculum was from agar and the subsequent ones from liquid culture to liquid culture in the log phase. Other experiments (not reported here)

showed no effect of the conditioned medium from shake flasks on the rate of growth of maize on agar under a variety of experimental conditions

Kinetin and zeatin

Nickell and Maretzki (200) found no stimulatory effects of kinetin at concentrations from 0.01 to 10 mg/litre on sugarcane cell suspensions. Kinetin caused an increase in dry weight of maize tissue at 0.01 ppm, whereas zeatin was inhibitory at 0.05 ppm or more and ineffective at lower concentration.

The lowest detectable concentrations of kinetin and zeatin in carrot root and soybean callus assays are about 5×10^{-9} M and 5×10^{-11} M respectively (147,172). There is no absolute requirement for these cytokinins in the case of maize and if they show any growth promotion it is at much lower concentrations than with many dicotyledonous tissues (Figures 9 and 10). The results with continuous subculture experiments (Table 46) show that zeatin may be stimulatory under certain conditions either due to adaptation to zeatin or selection of zeatin responsive cells. If cytokinins are required for these monocots, they are synthesized by the tissues themselves and no exogenous kinins are required. This appears to be true of all monocots which have been tested and true both for callus or roots of maize and wheat grown on agar or in shake flasks.

Diphenylurea and gibberellic acid

The effect of DPU is similar to that of kinetin, stimulation being observed at a low concentration (0.1 ppm); whereas higher concentrations are inhibitory. There is no absolute requirement of DPU for the growth of maize in shake flasks in long-term experiments,

though higher growth was observed as in the case of zeatin (Table 46), but only in the presence of NAA at 5 ppm and not 1 ppm. Further work on DPU is required at different NAA concentrations.

Gibberellic acid was inhibitory to maize, but further work with the cold-sterilized compound and with other gibberellins is needed. This hormone is, however, unnecessary for this tissue for survival and if required, is synthesized at a sufficient rate by the tissue.

Effect of growth retarding chemicals and abscisic acid

The effect of CCC and AMO-1618 is similar on both agar and shake flask cultures and the growth of maize is inhibited by these compounds. Abscisic acid is also inhibitory to maize both on callus-root mixtures on agar and root cultures in shake flasks. But the inhibition of the latter is much greater. When an inoculum obtained from shake flasks for growth under shake flask conditions was used, almost complete inhibition was observed in the presence of abscisic acid, whereas an inoculum from agar consisting of callus and roots was much less inhibited when grown in shake flasks (Tables 43 and 44). Further work is needed to confirm and extend the possible selective inhibition of root growth by this compound. This is the first report of such an effect and may be of physiological significance.

Naphthalene acetic acid concentration

Nickell and Maretzki (200) incorporated 2,4-D at 6 ppm in the medium for the growth of suspension cultures of sugarcane, whereas Gamborg and Eveleigh (79) added this auxin at 2 ppm for growing wheat and barley cultures in suspension culture. Trione *et al* (323) also obtained root development from callus tissue of wheat in liquid medium with low auxin concentrations.

The optimum concentration of NAA for maize grown in shake flasks was 5 ppm. Both lower and higher levels of this auxin caused a marked inhibition of growth (Fig.13). It is of interest that higher auxin levels inhibit root formation in agar cultures but even at 20 ppm maize grew only as roots in shake flasks.

Vitamins and glycine

Thiamine, nicotinic acid, pyridoxine and inositol were added as the vitamin supplements for growing wheat and barley cultures (79). Amino acids were supplied by N-Z Amine. The above vitamins except inositol in the yeast extract medium were also added for submerged suspension cultures of sugarcane cells (200). In the synthetic medium however a five vitamin mixture was used containing riboflavin and biotin in addition to thiamine, nicotinic acid and pyridoxine. Essentiality of these vitamins for different monocot tissues has not been determined.

Glycine is stimulatory but not essential for survival of maize (Tables 45 and 46). Nicotinic acid is probably not required. Further work on subculture of the tissue is needed to establish the essentiality of vitamins B_1 and B_6 for maize. They are probably the only vitamins needed by this tissue.

Nickell and Maretzki (200) studied the effect of inositol over a concentration range of 1 to 100 ppm but found no stimulatory effect of this substance on suspension cultures of sugarcane cells. Gamborg and Eveleigh (79) incorporated inositol at 100 ppm in their medium for growing wheat and barley cultures over long periods.

In contrast to the variable results with agar cultures, inositol showed stimulation of growth of the agitated maize cultures with an optimum at 100 ppm (Figure 15). However viable cultures were obtained even in the absence of inositol.

Effect of subculture

A tissue culture may be considered to be truly viable if it can be grown and subcultured indefinitely. Many of the cereal cultures in the literature were found to develop callus in the early stages soon after being isolated from the plant, but they failed to survive on subculture. From the results reported in Table 46 on long term growth of maize in different media, the following conclusions may be drawn regarding the growth of these cereals in agitated liquid cultures:

1) Viable cultures of maize, wheat and sorghum have been obtained which are capable of survival indefinitely on subculture.
2) These cultures are root cultures without any detectable callus (observed microscopically; washing of single roots from these cultures to remove any adherent callus did not affect their growth. Growth was therefore not due to a small amount of adhering callus).
3) The minimum requirements for survival of maize are in addition to basal salts, sucrose (or glucose), edamin (minus nitrate medium was not tested) and vitamins B_1 and B_6 and NAA. There is no requirement for cytokinin or DPU or glycine (nicotinic acid and inositol are possibly not required, but this remains to be established).
4) Higher phosphate (twice as high as in agar cultures) and 5 ppm NAA are stimulatory to growth.

The requirements for long term growth of wheat and sorghum cultures were found to be more or less identical with those of maize, though their rate of growth was less than that of maize.

Uptake of ions

The role of growth factors on the uptake of sugars, phosphate and organic nitrogen and the effect of different NAA concentrations on P_i uptake were studied in a few experiments (Table 47). These results should be regarded as preliminary and permit only tentative conclusions. The concentration of NAA was not very important for P_i uptake in the range of NAA and P_i concentrations which were studied for maize and wheat, whereas P_i concentration was more important in determining the amount of P_i taken up by the cells. It was not however determined whether at very low P_i auxin has an effect on the uptake of this anion (Tables 48 and 49).

With regard to the effect of hormones on maize it would be of interest to confirm that kinetin increases organic nitrogen and P_i uptake and that DPU reverses the effect of kinetin. These effects are complicated by the simultaneous growth of the tissue (Table 47). Short term experiments with labelled P_i at different concentrations and with different levels of P_i and DPU may throw light on the action of these compounds on ion movements into maize cells.

CHAPTER V

STUDIES ON DIFFERENTIATION

(Pages 181-199)

C O N T E N T S

SECTION 1

Studies on differentiation

Skoog (261) first showed that plant tissue cultures are useful for studying factors that regulate morphogenesis. Since then it has been repeatedly observed that the composition of the medium can play an important role in the control of differentiation (68,184,262,279).

With a view to inducing differentiation and studying the role of growth substances in this process the present studies were carried out on agar and shake flask cultures of maize and wheat. The results embodied in this Chapter were obtained with tissues which had been growing in culture over a number of years. There have been suggestions in earlier literature (15,318) that the capacity of plant cell cultures to form shoots decreased with the period of maintenance *in vitro* though there have also been reports, as with carrot cells studied by Steward and his colleagues (275,283) that they may retain the ability to differentiate even after long periods of growth *in vitro*.

a) Liquid media

As mentioned in the previous Chapter, one of the simplest methods for obtaining differentiation of maize, wheat and sorghum tissues into roots was by transference of the tissue grown on agar medium into liquid medium of the same composition. This effect was not noticed with rice tissue. The rate of agitation was unimportant since root differentiation occurred at speeds ranging from 1/6 to 200 rpm, but the tissues did not survive in stationary liquid cultures. This transformation into roots

was very striking with wheat tissues which grew mostly as undifferentiated callus on agar unlike maize and sorghum where the proportion of roots was higher. The capacity of these tissues to grow as roots in liquid medium was retained even after subculture for 3 to 5 years. Another striking feature regarding these cereal tissues was the reversibility of the differentiation into roots in liquid medium. Cereal cultures grown as roots in shake flasks over a number of passages when washed and transferred back to agar, reverted to their original morphological appearance of a mixture of callus and root. This reversible effect with maize, wheat and sorghum could be repeatedly demonstrated over a number of subcultures by alternate transfers from solid to liquid media. A microscopic examination of the cereal tissues on agar showed them to be a mixture of root and undifferentiated callus, whereas the root tissues in liquid did not show the adherence of any undifferentiated cells.

Since the only difference between the semi-solid and liquid media was agar, the possibility that this polysaccharide contained some substance(s) which gave rise to callus was taken into account. In separate experiments weighed amounts of agar (400 mg) were sterilized by autoclaving and added to cooled 50 ml liquid media in flasks which were later inoculated with maize tissue controls being kept without any added agar. After incubation for 18 days on a rotary shaker, there was no difference between the growth and appearance of the tissue in the presence or absence of agar.

In order to determine whether root formation was stimulated in agitated liquid cultures by higher oxygen levels, maize tissue was grown on agar medium in flasks having an inlet and outlet tube through which

oxygen was continuously passed, controls being kept in similar flasks with no oxygen. At the end of the growth period there was no difference in the morphological appearance between the controls and the oxygen treated tissues. In both there was a mixture of callus and root indicating that perhaps other factors in liquid cultures were responsible for the rooting response.

Effect of low concentrations of different auxins on maize and wheat in the presence of different compounds in agar cultures:

The media used in these experiments were composed mainly of the basal media for maize and wheat (BM_2) in which the respective auxins were added at 0.01 instead of 5.0 ppm NAA since a lower NAA concentration was found from earlier experiments to increase root formation. DPU was also omitted from the media. All the experiments reported in this Chapter were performed at a single auxin concentration, the other compounds also being tested at one level only and the results will be discussed on the basis of this auxin to growth substance ratio. It was not possible to study varying ratios of auxin to growth substance owing to limitations of space and time.

The inoculum used was from actively growing 30 day old maize or wheat tissues grown on their respective basal media (BM_2) for over 3 years and all these experiments were performed on semi-solid agar media. The tubes were illuminated intermittently with 12 hours light followed by 12 hours darkness with light from two 40-watt fluorescent tubes maintained at a height of 3 feet from the cultures. The temperature of the incubation chamber was 25°C$\pm$1°C and the humidity 60-70 per cent. The results are given as observations on the morphological appearance of the tissue as they appeared at the end of the growth period (which in this case was 70 days) since the purpose of these experiments

was not to study growth which, in many cases, was very poor, but to study differentiation. The results were drawn from the observations of 5 replicates in each experiment. Another object of these experiments was to see if shoot formation could be induced.

b) Low auxin

In this experiment the effect of 0.01 ppm of different auxins was tested on maize and wheat to see their effect on differentiation of these tissues. With maize tissue both aerial and ground roots 5 to 10 mm in length were formed in the absence or presence of any of the auxins (Table 50). Wheat tissue also formed roots in the absence of any auxin or with low concentrations of different auxins (Table 51), but in this case the roots grew downwards and developed laterals and root hairs. In all cases the roots were accompanied by small amounts of callus (Plate 7 shows the effect of low auxin concentration on wheat). Rice tissue also formed roots at low auxin concentration or in the absence of auxin (Plate 8).

c) Adenine

Skoog and Tsui (263) observed a promotion of shoot differentiation by adenine, sucrose and inorganic phosphate with tobacco tissue cultures. Adenine was tested on maize and wheat tissues at a concentration of 20 ppm and in the presence of different auxins added at low levels of 0.01 ppm to see its effect on morphogenesis in these tissues. With maize tissue (Table 52) grown in the presence of adenine without auxin or with low levels of auxin, the tendency to form aerial roots (Table 50) was suppressed and instead a few long roots growing downwards developed. With α-NAA the roots also showed laterals.

TABLE 50

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON MAIZE TISSUE

Basal medium: BM_2

Changes: NAA omitted and substituted by respective auxins at 0.01 ppm. DPU omitted.

Period (days): 70

+ , presence of at least 40–50 mg callus

	Auxin	Callus	Observations
1	No auxin	+	5–10 aerial roots 2–5 mm long and 3–5 roots growing into medium (5–10 mm long).
2	2,4-D	+	Root formation as in (1)
3	α-NAA	+	Root formation as in (1), but the number of roots growing into the medium was 5 to 7.
4	2,4,5-TCP	+	" " "
5	IAA	+	" " "

TABLE 51

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON WHEAT TISSUE

Basal medium: BM_2

Changes: NAA and DPU omitted. Respective auxins added at 0.01 ppm.

Period: 70 days.

+ , presence of 40–50 mg callus

	Auxin	Callus	Observations
1	No auxin	+	3–5 long roots (10–15 mm) with small laterals (5–10 mm) and root hairs growing into the medium
2	2,4-D	+	Root formation as in (1)
3	α-NAA	+	" " "
4	2,4,5-TCP	+	" " "
5	IAA	+	" " "

PLATE 7: WHEAT CULTURES

(1) 0.01 ppm NAA

(2) 0.01 ppm IAA

(3) 0.01 ppm 2,4-D

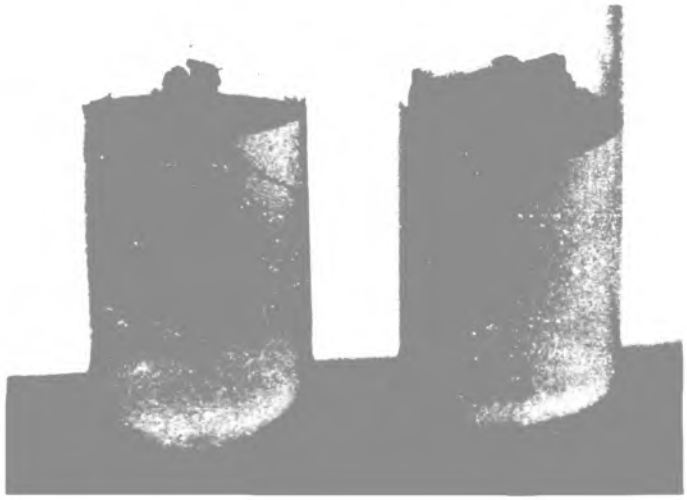


PLATE 8: RICE CULTURES

(1) 0.01 ppm NAA

(2) 5.0 ppm NAA

With wheat tissue (Table 53) adenine tended to increase the length of the roots in the absence of auxin or when α-NAA, 2,4,5-TCP or IAA were present in the medium. In the presence of adenine and 2,4-D the roots that were formed were thick and short. These results may be compared with the results given in Table 51 for wheat, where the medium differs only in the adenine content. In all cases with maize and wheat, callus was also present in a small amount. No shoot formation was observed in any of the tubes.

d) Kinetin

Skoog and Miller (262) showed that a balance of auxin and kinetin determined morphogenetic patterns in tobacco callus cultures. A relatively high auxin/low kinin ratio stimulated rooting and the reverse produced shoots. Since the purpose of this experiment was to induce shoot formation in maize and wheat tissues, kinetin was added at the high concentration of 5 ppm, with low levels of the different auxins.

Kinetin at the concentration tested markedly suppressed growth of maize tissues, the only growth being the induction of 1 or 2 thick, black and short aerial roots. This effect was noticed in the absence of any auxin or at the low auxin level tested (Table 54).

With wheat tissues also the roots that developed were thick, dark coloured and short, but in this case some roots showed negative whereas others showed positive geotropism (Table 55)in the absence of auxin or with NAA. With 2,4-D, 2,4,5-T and IAA the roots increased in number and thickness.

These results with maize and wheat suggest that other kinetin concentrations require to be tested. A small amount of callus was also present in all these cultures.

TABLE 52

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON MAIZE TISSUE ON AN ADENINE CONTAINING MEDIUM

Basal medium: BM_2

Changes: NAA and DPU omitted. Respective auxins added at 0.01 ppm and adenine at 20 ppm.

Period: 70 days

+ , presence of 40–50 mg callus

	Auxin	Callus	Observations
1	No auxin	+	3–5 long roots (10–15 mm) (growing into the media)
2	2,4-D	+	Root formation as in (1)
3	α-NAA	+	Root formation as in (1) but with laterals
4	2,4,5-TCP	+	Root formation as in (1)
5	IAA	+	" " "

TABLE 53

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON WHEAT TISSUE ON AN ADENINE CONTAINING MEDIUM

Basal medium: BM_2

Changes: NAA and DPU omitted. Respective auxins added at 0.01 ppm and adenine at 20 ppm.

Period: 70 days.

+ , presence of 40–50 mg callus

	Auxin	Callus	Observations
1	No auxin	+	Many thin and long roots (20–25 mm), having laterals, growing into the medium.
2	2,4–D	+	2 to 3 roots formed which were thick and growing into the medium.
3	α–NAA	+	Root formation as in (1) but the number of roots less.
4	2,4,5–TCP	+	Root formation as in (3).
5	IAA	+	" " "

TABLE 54

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON MAIZE TISSUE ON A KINETIN CONTAINING MEDIUM

Basal medium: BM_2

Changes: NAA and DPU omitted. Respective auxins added at 0.01 ppm with kinetin at 5 ppm.

Period: 70 days.

+ , presence of 40–50 mg callus

	Auxin	Callus	Observations
1	No auxin	+	Tissue black, with 1 or 2 thick black aerial roots, 3–5 mm long
2	2,4–D	+	Root formation as in (1)
3	α-NAA	+	" " "
4	2,4,5–TCP	+	" " "
5	IAA	+	" " "

TABLE 55

EFFECT OF LOW CONCENTRATIONS OF DIFFERENT AUXINS ON WHEAT TISSUE ON A KINETIN CONTAINING MEDIUM

Basal medium: BM_2

Changes: NAA and DPU omitted. Respective auxins added at 0.01 ppm with kinetin at 5 ppm.

+ , presence of 10-20 mg callus

	Auxins	Callus	Observations
1	No auxin	+	Tissue black. 3 to 5 thick and short roots (aerial and downward) 3-5 mm long with root hairs formed.
2	2,4-D	+	The number of roots formed are more than in (1) and thicker.
3	α-NAA	+	Root formation as in (1)
4	2,4,5-TCP	+	Root formation as in (2)

e) Gibberellic acid (GA_3) and naphthalene acetic acid

In the basal medium (BM_2) used in this experiment, DPU was also incorporated unlike in the previous studies where the effect on differentiation was studied in its absence. The effect of GA_3 and NAA were studied both in agar and in liquid media in shake flasks with wheat tissue and were tested at one concentration only of 1 ppm of GA_3 and 2 ppm of NAA (Table 58). Gibberellic acid showed a very distinct capacity to initiate roots on wheat tissue in agar media. Though the tissue grown with 2 ppm NAA showed very slight root formation, GA_3 had the ability to convert a part of the callus mass into short roots. In shake flasks the tissue appeared mostly as roots even without GA_3 and no change in the number/or in appearance of roots was observed. Maize tissue also behaved in a similar manner to wheat tissue in its response to gibberellic acid towards root formation.

f) Naphthalene acetic acid concentrations

In this set of experiments as in the previous one, the basal media (BM_2) also contained DPU and NAA at different concentrations ranging from 0 to 20 ppm in order to study the effect of varying concentrations of NAA on root formation when DPU was present at 1 ppm. From Table 59 it is clear that if NAA is omitted from the agar medium or is added at very low concentrations root formation was favoured whereas high auxin concentrations suppressed root growth. In liquid grown cultures only root formation occurred whether auxin was absent from the medium or added at levels upto 20 ppm. The only observation of interest was that the roots were thicker and shorter at higher concentrations.

TABLE 56

EFFECT OF NAPHTHALENE ACETIC ACID AND GIBBERELLIC ACID ON WHEAT TISSUE GROWN ON AGAR OR LIQUID MEDIA

Basal medium: BM_2

Changes: NAA at 2 ppm and GA (1 ppm) when added. Alternating 12 hour periods of light and darkness. Liquid cultures in shake flasks.

Period: 70 days

	Condition of medium Agar or liquid	NAA or GA added	Observations
1	Agar	-	Forms few aerial roots (2–5 mm) and 4 to 5 slightly thick roots (5–10 mm) growing downwards. Also a little callus.
2	Liquid	-	Grows as long and thin roots (20–30 mm and over).
3	Agar	NAA	Mostly callus with tiny roots on the surface.
4	Liquid	NAA	Mostly roots develop, 10–20 mm, and thick.
5	Agar	GA	Forms 5 to 10 thin and long roots (5–10 mm) which grow downwards and also a few aerial roots (2–5 mm).
6	Liquid	GA	Forms many thin long roots 20–30 mm and over.
7	Agar	NAA+GA	Both aerial and ground roots develop (2–5 mm long).
8	Liquid	NAA+GA	Thin and long roots, 10–20 mm long.

TABLE 57

EFFECT OF DIFFERENT CONCENTRATIONS OF NAPHTHALENE ACETIC ACID ON ROOT FORMATION IN MAIZE TISSUES IN AGAR OR LIQUID MEDIA

Basal medium: BM_2

Changes: NAA at different concentrations. Alternating 12 hour periods of light and darkness. Liquid cultures in shake flasks.

Period: 70 days.

	NAA concentration	Condition of medium Agar or liquid	Observations
	ppm		
1	0	Agar	4 to 5 each of aerial and ground roots, 5-10 mm long, together with a little callus.
2	0	Liquid	Forms a number of long thin roots (20-30 mm) with laterals (10-15 mm).
3	0.1	Agar	Roots are less and callus is more than in (1).
4	0.1	Liquid	Root formation as in (2).
5	1.0	Agar	Few aerial roots (1-2 mm) together with callus.
6	1.0	Liquid	Root formation as in (2)
7	5.0	Agar	Mostly callus with 2-3 aerial roots(1-2 mm).
8	5.0	Liquid	Length of roots shorter than in (2) (10-15 mm long) with short laterals (5 mm long).
9	20.0	Agar	Mostly callus is formed. Tissue differentiation is reduced.
10	20.0	Liquid	Root formation takes place, but the roots are thick and growth is inhibited.

SECTION 2

Discussion

During the last 25 years callus cultures of plants have been used increasingly for investigating factors regulating plant organ formation. Tissue culture methods offer better control of nutritional and environmental factors in contrast to the conventional methods in which cuttings etc. are used.

After the studies of Skoog (261) and his coworkers (262) referred to earlier, differentiation has been demonstrated in endive and parsley (335,336), carrot (278), geranium (64), Convolvulus (68) and in many other tissues. Plants were also obtained from callus tissues from single carrot cells in suspension (98,283) and from single isolated cells of tobacco (62,337) illustrating the totipotency of individual cells.

Organ differentiation has also been reported from callus tissues of monocotyledonous plants; from asparagus (285,360), orchid (231,287), rice (205,206), wheat (79,258,323), sorghum (163), oat (58), lilium (257) and gladioli (369).

The discussion in this Chapter will be mainly with reference to the earlier work carried out on organogenesis of monocots, as it is beyond the scope of this discussion to give a detailed survey of the extensive literature on differentiation in plant tissue cultures.

Liquid cultures

Trione et al (323) observed that when somatic wheat callus grown on Hildebrandt's "D" medium (110) was subcultured into Torrey and Reinert's (320) suspension culture medium - a chemically defined medium with a very

low auxin level - either agar or liquid, the callus formed many roots. Continued growth in this medium produced a branching and enlarging root system with very little proliferation of callus. Gamborg and Eveleigh (79) also found that when newly established cultures of wheat and barley were transferred to liquid medium they frequently differentiated and produced roots, especially when 2,4-D was replaced by IAA or NAA. These cells however later lost their ability to produce roots under the same conditions; the same growth regulators used earlier for root production later produced suspension cultures.

Torrey (318) studied the effect of a prolonged period of subculture on the capacity of Pisum sativum L, callus tissue to initiate roots and found that there was a progressive loss of organ forming capacity in all tissues growing over long periods of subculture. By chromosomal analysis it was observed that initially all callus tissues could be stimulated to form normal diploid roots. After several years of continuous subculture some callus tissues formed normal tetraploid roots. Still later these callus tissues lost completely their capacity to initiate roots. This loss was paralleled by increasing abnormalities in the chromosomal numbers and greater frequency of aneuploidy. Muir (187a) also reported that in tobacco callus tissues of single cell origin, those strains which seemed less able or unable to form buds in culture were polyploid strains. Barba and Nickell (15) studied the effect of age on organogenesis in tissue cultures of commercial sugarcane cultures. Clones established from four sugarcane cultivars and subcultured for five years and new isolates from the same cultivars were used in these studies. Shoots were obtained readily from three of his fresh isolates under the same treatments which were ineffective for five year old isolates.

The cereal cultures reported in this thesis showed differentiation to roots on transfer to agitated liquid cultures, but this effect differs from

earlier work in being demonstrable even after prolonged culture *in vitro* for several years. It was also unique in the ready reversibility of this phenomenon, the change from callus to roots or from roots to callus occurring on transfer to agitated liquid or agar media. This is the only report of such reversibility in plant cultures as far as the author is aware. The reason for this differentiation is not known. Availability of oxygen to all parts of the inoculum may be a factor in liquid medium, but oxygen had no effect on agar cultures.

White (350) and Skoog (261) also showed that callus tissue derived from the tobacco hybrid *Nicotiana glauca* X *Nicotiana langsdorffii* remained uniformly parenchymatous on an agar medium but underwent differentiation when transferred to liquid medium. However in most cases plant formation was obtained using agar media (262,278).

Low auxin concentration

Shimada *et al* (258) studied the effect of different concentrations of 2,4-D and IAA on organ redifferentiation from callus induced from seedling roots of common wheat. Media containing no auxin or 2,4-D at concentration less than 1 ppm consistently caused root formation with occasional formation of shoots. Root formation also took place with IAA concentrations from 0.5 to 50.0 ppm. Nishi *et al* (206) transferred pieces of third generation rice callus into medium lacking 2,4-D and obtained redifferentiated tissues with shoots and roots. Barba and Nickell (15) tried various concentrations of NAA and 2,4-D on callus clones established from 4 sugarcane cultivars and subcultured for 5 years using the media of White (352) and that of Murashige and Skoog (193). Shoot formation was not induced in any of these long-established cultures.

When different auxins were tested on maize and wheat at low concentrations of 0.01 ppm on the basal medium containing edamin, only root development was observed in all cases (Table 50 and 51). The proportion of

callus was less in the absence of any auxin than in the presence of these growth substances.

The results obtained with different NAA concentrations showed that increased auxin favours callus formation on agar but had no effect in liquid cultures (Table 57).

The literature on the effect of kinetin and adenine on plant cells will not be reviewed since their effect on cereal cultures was not very marked.

Gibberellic acid and NAA

Gautheret (90) studied the effect of GA_3 on root formation in Jerusalem artichoke tissues. He found marked rooting properties for GA_3 when associated with NAA (10^{-7}M) and kept in the dark. At high NAA (10^{-5}M) concentrations, the minimum amount of GA_3 capable of showing rooting activity was very low viz., from 10^{-12} to 10^{-11}M. Murashige (190) described inhibition of shoot formation in tobacco tissue by GA_3 and later (191) showed that gibberellin in concentrations which stimulated growth of tobacco callus inhibited root and shoot formation. The inhibitory effect on shoot formation was common to gibberellins A_1, A_3, A_4, A_5, A_7, A_8 and A_9, which were tested at a concentration of 3×10^{-7}M.

Gibberellic acid (GA_3) (1 ppm) was found to have marked root forming capacity on wheat and maize tissues in agar cultures when added without NAA or with 2 ppm NAA (Table 56).

Though many recent reports are available on shoot formation from callus cultures of the monocots, most organogenesis in this group has been achieved from first to fifth generation cultures. Exceptions to this are the monocot lilium (257) with which plant formation took place even after 7 subcultures; sugarcane which showed root formation even after 5 years, the chemical 2,2 dichloropropionic acid (dalapon) increasing this effect (15); wheat grown in shake flasks on Torrey and Reinert's medium (320) with low

auxin levels (323); asparagus and orchid by the use of a sequential procedure, by changing or omitting sequentially with each subculture, cocomut milk or the auxin in the basal medium (287).

Plant roots and shoots respond to gravity and light in a definite way. There are typical geotropic and phototropic responses for roots and shoots. Khan (126) observed that when seedlings of monocots or dicots were grown in the presence of morphactins n-butyl-9-hydroxy fluorene (9) carboxylate and methyl-2-chloro-9-hydroxy fluorene (9) carboxyl in a vertical or horizontal plane, the roots and shoots lost their capacity to respond to gravity or to unilateral light sources. The morphactins were isolated by Schneider et al (249) and considered to be a novel group of plant growth retarding substances having interesting effects on morphogenesis. Juniper et al (121) have drawn attention to the importance of the root cap in the geotropic response of Zea mays roots. They found that removing the cap abolished the downward curvature of horizontally placed roots even though the roots continued to elongate. Gibbons and Wilkins (93) in studies with Zea mays roots found the root cap to be the source of at least one growth inhibiting substance, which was responsible for the geotropic response of the root.

Tissue cultures of maize and wheat also exhibited various geotropic responses to various media. The reason for these variations in geotropism in the tissues is not known, though even the aerial roots were found on examination to have root caps.

Some of the factors which were effective in enhancing root formation may be summarized as (1) agitation in liquid medium (2) addition of gibberellic acid and (3) very little or no auxin. No shoot formation was observed in any of the experiments.

CHAPTER VI

CHEMICAL STUDIES ON MORPHOGENESIS AND GROWTH

(Pages 200-211)

C O N T E N T S

S E C T I O N 1

Chemical studies on morphogenesis and growth

A comparison of the chemical composition and enzymic pattern of maize and wheat cells grown on agar and in liquid cultures was made in order to find out whether there were any significant differences between the cultures consisting of roots or callus. For this purpose extracts were separately prepared from the callus and root cultures and a comparison was made of a few of the enzymes of the glycolytic and hexose monophosphate pathways and citric acid cycle and a few hydrolytic enzymes. The fresh weight and dry weight, cell number, RNA and DNA content of maize, wheat and rice tissues at different periods of growth on agar media were also determined.

a) E n z y m e s

Preparation of extracts: All the operations described below were carried out at 0°. Four to five grams of actively growing maize or wheat tissues in the mid-logarithmic phase from agar and liquid media (BM_2P) were pressed gently between filter papers to remove adhering medium and the root outgrowths appearing from agar cultures were removed as far as possible. The tissue was then ground in a chilled mortar with 0.05 M potassium phosphate buffer, pH 7.5 (2 ml per g of tissue) till samples of the extract on microsopic observation showed very few intact cells (5 to 10 per field with 100 times magnification). The crude extract was then centrifuged at 2500 rpm for 10 minutes, the residue suspended in 2 ml of the buffer and recentrifuged at the same speed for 5 minutes. The two supernatant liquids were combined and centrifuged in a Spinco preparative ultracentrifuge at 105,000 x g for 30 minutes. The volume of the supernatant was noted after centrifugation and the clear supernatant then

dialyzed against two changes of 100 volumes of 0.05 M phosphate pH 7.5 buffer with stirring for 18 hours to remove endogenous substrates. The volume after dialysis was noted and the enzyme assays were run with the dialyzed extract. All the enzyme assays were completed in as short a time as possible after the preparation of the dialyzed enzyme. However no decrease in the activities of the enzymes could be detected in extracts stored at -20°C and tested even after a week of extraction indicating the stability of these enzymes on storage. Moreover, enzyme activities in duplicate runs agreed fairly well.

Unit of activity

The unit of activity of esterase is defined as the umoles of p-nitrophenol released in 60 minutes, of α-amylase as the mg of maltose released in 3 minutes, of β-glycerophosphatase as the ug of phosphorus liberated per minute. For deoxyribonuclease and ribonuclease the unit of activity is defined as the amount of enzyme capable of bringing about an increase in optical density at 260 mμ of 1.0 for a light path of 10 mm per 10 minutes under the conditions of the experiment. In the case of all the other enzymes it is defined as the amount of enzyme required for the reduction of 1 umole of TPN or oxidation of 1 umole of DPNH under the assay conditions.

The methods followed for the estimation of the enzymes are given in Chapter I.

Enzymes: Most of the enzymes were present in similar amounts in maize roots and callus. The activity per mg protein was taken as the basis for comparison. These include alcohol dehydrogenase, hexokinase, phosphoenol-pyruvate carboxylase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, β-glycerophosphatase and α-amylase. Root tissue had a higher content of glucose 6-phosphate dehydrogenase, malate dehydrogenase and malic enzyme and less of ribonuclease and pyruvate kinase. The most striking

TABLE 58

TOTAL ACTIVITIES OF CERTAIN ENZYMES OF MAIZE GROWN ON AGAR AND LIQUID MEDIA

Protein: Callus 64.9 mg/g dry tissue
Root 95.2 mg/g dry tissue

	Enzyme	Activity			
		Callus	Roots	Callus	Roots
		units/mg protein		units/g dry tissue	
1	Lactate dehydrogenase	Not detectable	0.009	Not detectable	0.8
2	Pyruvate kinase	0.025	0.011	1.6	1.0
3	Alcohol dehydrogenase	0.063	0.083	4.1	7.8
4	Hexokinase	0.004	0.003	0.3	0.3
5	Phosphoenol pyruvate carboxylase	0.125	0.155	8.1	14.7
6	Isocitrate dehydrogenase	0.109	0.100	7.1	9.5
7	Malate dehydrogenase	2.800	4.200	182.0	399.0
8	Malic enzyme	0.042	0.075	2.7	7.1
9	Glucose 6-phosphate dehydrogenase	0.042	0.090	2.7	8.5
10	6-Phospho gluconate dehydrogenase	0.009	0.009	0.6	0.8
11	Deoxyribonuclease	Not detectable	trace	Not detectable	trace
12	Ribonuclease	6.700	3.100	435.0	294.0
13	β-glycerophosphatase	0.050	0.053	3.3	5.0
14	α-amylase	0.250	0.323	16.0	30.0
15	Esterases	2.120	9.900	137.0	940.0

TABLE 59

TOTAL ACTIVITIES OF CERTAIN ENZYMES OF WHEAT GROWN ON AGAR AND LIQUID MEDIA

Protein: Callus 63.3 mg/g dry tissue
Root 52.5 mg/g dry tissue

	Enzyme	Activity			
		Callus	Roots	Callus	Roots
		units/mg protein		units/g dry tissue	
1	Lactate dehydrogenase	Not detectable	trace	Not detectable	trace
2	Pyruvate kinase	0.093	0.016	5.8	0.8
3	Alcohol dehydrogenase	0.053	0.059	3.3	3.0
4	Phosphoenolpyruvate carboxylase	0.144	0.140	9.0	7.3
5	Isocitric dehydrogenase	0.048	0.115	3.0	5.9
6	Malate dehydrogenase	2.960	4.250	186.0	221.0
7	Malic enzyme	0.006	0.042	0.4	2.2
8	Glucose 6-phosphate dehydrogenase	0.037	0.120	2.3	6.2
9	6-phospho gluconate dehydrogenase	0.010	0.008	0.6	0.4
10	Deoxyribonuclease	Not detectable	trace	-	-
11	Ribonuclease	Not detectable	3.300	Not detectable	171.0
12	β-glycerophosphatase	0.100	Not detectable	6.3	Not detectable
13	α-amylase	Not detectable			
14	Esterases	1.480	1.020	93.2	53.0

difference was in the esterase content of roots which was nearly 4 to 5 times higher than that of callus. Lactate dehydrogenase was not detectable in callus but was present in roots. The relatively high malate dehydrogenase and the barely detectable deoxyribonuclease of both callus and roots of maize and wheat are noteworthy (Table 58).

Both wheat callus and roots contained similar amounts of alcohol dehydrogenase, phosphoenolpyruvate carboxylase, 6-phosphogluconate dehydrogenase and esterase. The deoxyribonuclease and α-amylase contents both were negligible. Lactate dehydrogenase was negligible in callus and only traces were present in roots. In wheat tissue, roots contained higher isocitrate dehydrogenase, malate dehydrogenase, malic enzyme, glucose 6-phosphate dehydrogenase and ribonuclease than callus and less pyruvate kinase and β-glycerophosphatase (β-glycerophosphatase was not detectable in roots and ribonuclease in callus) (Table 59).

b) S u g a r s

Callus and root tissues of maize and wheat grown on the basal medium BM_2P on agar and in shake flasks in the mid logarithmic phase were removed, weighed and extracted 3 times with hot 70 per cent alcohol. The 70 per cent alcohol extract was then treated according to the methods given in Chapter I for the isolation and identification of the free sugars.

Free sugars: Of the free sugars, only sucrose, glucose and fructose were chromatographically detectable in maize callus or roots. There was no significant difference in the sucrose content, but there was a slightly greater amount of monosaccharides in the callus than in roots on a dry weight basis. It is doubtful whether this is significant. The amounts of the three sugars were nearly the same (Table 60).

Wheat also contained only the same three carbohydrates in callus or roots. On a fresh weight basis, roots contained much more fructose than

TABLE 62

CORRELATION OF THE GROWTH OF MAIZE, WHEAT AND RICE WITH REGARD TO DRY WEIGHT, CELL NUMBER, RNA AND DNA WITH TIME

Tissue	Age of tissue	Dry weight	Cell number per mg wet tissue	Cell number per g dry tissue	RNA	DNA
	days	mg/g wet tissue	(in thousands)	(in millions)	mg/g dry tissue	mg/g dry tissue
Maize	18	84	8.4	101	36	1.8
"	25	70	7.5	105	38	2.5
"	32	95	8.8	88	36	1.7
"	40	90	9.5	104	27	1.8
Wheat	18	61	7.9	126	17	1.9
"	25	61	7.9	125	24	2.0
"	32	62	7.3	116	25	1.4
"	40	73	6.8	95	19	1.7
Rice	18	114	9.9	90	25	1.8
"	25	98	11.9	119	30	1.9
"	32	95	11.5	115	28	2.1
"	40	91	12.3	123	25	2.0

callus and fructose formed the major sugar in wheat and constituted over half the total carbohydrate of wheat cultures (Table 61).

c) RNA, DNA and cell counts

DNA and RNA estimations and cell counts were carried out on maize, wheat and rice tissues grown on their respective basal media containing agar (BM_2 for maize and wheat and BM_3 for rice) and harvested at 18, 25, 32 and 40 days. The methods used are given in Chapter I.

The number of cells per g dry tissue of maize and wheat was more or less the same during the entire period whereas rice showed a gradual increase in cell number upto the 40th day. The RNA and DNA contents per g of dry tissue of the three cereals did not show very marked increases or decreases between the 18th and 40th day when compared on a per g dry basis (Table 62). However cell counts and DNA and RNA contents during the first week of growth may show a different picture.

SECTION 2

Discussion

Though the literature on the metabolism and enzymes of plant tissues is meager compared to that on animal tissues and microorganisms, several studies on plant cultures have been reported in recent years. A list of the intra or extracellular enzymes detected in cultured plant tissues has been given by Krikorian and Steward (135). Comparative studies of the metabolic activity of normal and crown gall tissues were made by Lance (139,140,141) and Tamaoki *et al* (311,312,313). Tumour tissue had less cytochrome oxidase, but more malate and succinate dehydrogenases per unit of protein nitrogen of mitochondria from callus tissue of *Scorzonera* and *Helianthus tuberosus* L (140). The respiration of the tumor tissue was less sensitive to the inhibitors; cyanide, sodium azide and carbon monoxide. Tamaoki *et al* (311,312,313) compared the respiratory metabolism of mitochondria isolated from callus and crown gall tissue cultures of tomato. The mitochondria from normal and tumour tissue of tomato differed quantitatively but not qualitatively in their oxidative and phosphorylative activities. Mitochondria from crown gall tissue cultures oxidized intermediates of the Krebs cycle more slowly but oxidized ascorbic acid more rapidly than callus mitochondria. Srivastava *et al* (267) found that only tumour tissue and not normal tissue of *Rumex acetosa* contained the enzyme nicotinamide amidohydrolase.

Parekh *et al* (222) compared the enzyme composition of excised roots of *Phaseolus mungo* cultivated *in vitro* with that of the roots grown *in vivo* over a 7-day growth period. The enzyme activities when expressed in terms of tissue nitrogen were generally found to decrease

during the course of cultivation *in vitro*. However the activities in the case of fumarate hydratase as well as of most of the dehydrogenases were restored to the original levels by the seventh day. Similar changes were also found during cultivation of the roots *in vivo*. The changes in enzyme activity during *in vivo* and *in vitro* cultivation over a 7-day growth period were generally in the same direction except that a difference was observed in the case of fumarate hydratase. Verma and van Huystee (339) observed quantitative and qualitative differences in peroxidase isozymes between groups of cells of different sizes from a suspension culture obtained from peanut cotyledons. The appearance of 2 new peroxidase isozymes was correlated with cellular differentiation and the enlargement of the cells from 0,5 to 4,0 mm in diameter.

The higher content of isocitrate dehydrogenase, malate dehydrogenase, glucose 6-phosphate dehydrogenase and lower content of pyruvate kinase in roots in comparison with callus is common to both wheat and maize. The relatively higher esterase of maize roots was not observed with wheat, and wheat and maize also differed in their relative amounts of ribonuclease in root and callus. The differences in enzyme content of callus and root tissues of maize and those of wheat show some resemblances. However the enzyme content of the medium was not determined and it is possible that the medium may contain some enzymes. Further work with other monocot cultures are needed to find out whether the observed differences are common to all such cultures and whether they are of significance in differentiation.

The carbohydrates of the tissues were examined mainly to determine whether any sugars other than glucose, fructose and sucrose were present in callus or roots of maize and wheat. No other sugars were present in detectable amounts. The high fructose content of wheat is of interest

and the effect of different experimental conditions on the relative amounts of the three carbohydrates needs to be studied to establish the significance of this observation.

Parekh *et al* (222) found that roots of *Phaseolus mungo* grown *in vitro* contained 13.2 mg RNA and 0.14 mg DNA per 100 mg dry tissue at the commencement of growth, the levels on the 7th day being 8.6 mg RNA and 0.25 mg DNA, whereas on the 7th day roots of the same plant cultivated *in vivo* contained 5.0 mg RNA and 0.194 mg DNA.

Steward *et al* (284) studied the changes in cell number, DNA and RNA contents of cultured plant cells over a 14 day growth period on two different media. The total number of cells of explants grown on a simple basal medium varied from 8000 per mg wet tissue on the first day to about 5000 at the end of 14 days. The DNA and RNA contents were 0.2 ug and 0.1 ug/mg wet tissue at the beginning, but increased to 0.35 and 1.1 ug after 14 days. When the medium was supplemented with CM and casein hydrolyzate the cell number of the explants increased from 8000 to 23,000 by the 8th day and then gradually decreased till the 14th day. The DNA and RNA contents increased from 0.2 and 0.1 ug/mg wet tissue on the first day to 0.4 and 2.1 ug/mg wet tissue on the 4th and 6th days respectively and then showed a drop till the 14th day.

Arya *et al* (10) recorded the average number of cells per mg fresh weight of 6 clones of *Phylloxera* gall and normal origin. The cell count was highest in all cases on the 10th day, the number of cells varying from 8000 to 20,000 in the different clones. At the end of 40 days the cell number dropped to about 5000 to 12,000. Arya (9) studied the RNA and DNA contents of the six normal and crown gall clones on different media. The RNA content in general of the 6 different clones varied

between 2 and 9 mg per g dry tissue on the 10th day, decreasing to about half this amount at the end of 40 days. The DNA contents similarly varied from 0.9 mg to 2.0 mg/g dry on the 10th day, with each of the clones, and showed a gradual reduction with the growth of the tissues.

The three cereals, maize, wheat and rice, contained between 7000 and 12,000 cells per mg fresh tissue. The change in cell number, DNA and RNA contents was very slight between 18 and 40 days. for maize and wheat. Rice tissue however showed a slight increase in cell number between 18 and 40 days.

CHAPTER VII

SUMMARY AND CONCLUSIONS

(Pages 212-219)

SUMMARY AND CONCLUSIONS

1. The conditions for initiating callus cultures from root or epicotyl segments of four monocotyledonous plants, Zea mays (maize), Triticum vulgare (wheat), Oryza sativa (rice) and Sorghum vulgare (sorghum) seedlings were determined. These cultures were maintained in vitro for periods ranging from 3 to 5 years by subculture every 45 days and may be regarded as viable cultures of these plants. The cultures from corn and sorghum represent the first viable cultures obtained from these two monocotyledonous plants as far as the author is aware.

2. For the initiation of callus from these cereals, an agar medium containing either modified Smith's or White's mineral salts, vitamins and sucrose supplemented with naphthalene acetic acid was sufficient, although additional supplementation with edamin, diphenyl urea, inositol and higher naphthalene acetic acid levels gave better callus formation of maize, wheat and sorghum tissues. Rice tissue, however, formed better callus on further supplementation of the above medium with adenine and guanine and/or cytidylic acid or tyrosine.

3. Maize and sorghum cultures grown on agar medium consisted of a mixture of callus and roots. Wheat consisted almost entirely of callus with only a few roots in the later stages of growth. Rice consisted only of callus. These cultures retained this characteristic appearance even after many years in culture.

4. In long term experiments over a period of 3 to 5 years a medium consisting of mineral salts, vitamins, sucrose, edamin (or casein hydrolyzate) and naphthalene acetic acid was adequate for the growth and survival of maize tissue on repeated subculture. Inositol, diphenylurea, adenine, guanine or ammonium salts singly or in different combinations were not found to be essential for the long term survival of maize cultures. The overall growth was, however, in some cases markedly improved in the presence of some of these supplements.

5. Starch or glucose instead of sucrose was also adequate for the survival of maize tissue on subculture in long term experiments.

6. Wheat and sorghum tissues could be maintained by sub-culture on a modified Smith's mineral salt-vitamins-sucrose medium supplemented with edamin, inositol, diphenyl urea and a high level of naphthalene acetic acid. For the maintenance of rice cultures the above medium was supplemented with adenine and guanine, and/or cytidylic acid or tyrosine. Starch could replace sucrose as a carbon source for sorghum tissue. It was, however, not definitely established whether diphenyl urea and the other supplements were essential for the survival of these three cereal cultures in vitro.

7. All the four cereal cultures grew very poorly during the initial stages, but the growth rate improved markedly on repeated subculture.

8. All the four cereal tissues were grown on five basal salts media (modified White's, Smith's, Murashige and Skoog's, Hildebrandt's and Knop's) which varied in their effectiveness in

promoting growth. A modified Smith's basal medium gave good growth of all the tissues.

9. Nitrate or ammonium nitrate or edamin as sole sources of nitrogen supported growth of maize, wheat and sorghum tissues. Maize tissue grew well on urea also. Growth in the presence of other ammonium salts as sole source of nitrogen was very poor or negligible. Supplementation of the basal nitrate medium with 0.05 to 0.1 per cent edamin was stimulatory for maize, wheat and rice tissues.

10. All four cultures grew well on sucrose or glucose. The Growth of maize, wheat and rice was also supported by fructose or maltose while wheat tissue grew well also on glycerol. All the tissues grew on starch. Xylose, lactose and galactose were ineffective.

11. The optimum sucrose concentration for rice tissue was 2 per cent and the optimum maltose concentration was 1 to 3 per cent.

12. The mixture of thiamine, pyridoxine, nicotinic acid and glycine (White's B solution) gave maximum growth of maize, wheat and rice tissues when added at a level, 5 to 10 times higher than that in White's medium.

13. Diphenyl urea stimulated the growth of maize at 0.001 ppm, of rice at 0.01 ppm and of wheat over a broader concentration range.

14. Coconut milk and kinetin showed little or no stimulation of growth of maize, wheat and rice, and were inhibitory at higher concentrations.

15. The effect of zeatin was variable, being stimulatory for maize, inhibitory for wheat after the second subculture and without any effect on sorghum.

16. Maize, wheat and rice showed inhibition of growth even at low levels of GA_3. The different gibberellins (GA_1 to GA_{13}) inhibited growth of maize and wheat, except for GA_4 which stimulated growth of wheat.

17. The auxins which promoted growth of the different tissues were as follows in decreasing order of effectiveness:-

Maize (indole acetic acid, 1-naphthalene acetic acid, 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid).

Wheat (1-naphthalene acetic acid, indole acetic acid, indole propionic acid, indole butyric acid, 2-naphthalene acetic acid).

Rice (2,4-dichlorophenoxyacetic acid, indole butyric acid, 1 and 2-naphthalene acetic acids, indole propionic acid, indole acetic acid).

Sorghum (2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, 1 and 2-naphthalene acetic acids, indole propionic and indole acetic acid).

An auxin was essential for the growth of the cereals.

18. The optimum naphthalene acetic acid concentration was between 5-10 ppm for all the cereals.

19. (2-chloroethyl) trimethyl ammonium chloride (CCC) and 4-Hydroxyl-5-isopropyl-2-methyl phenyl trimethyl ammonium chloride, 1-piperidine carboxylate (AMO-1618) showed marked inhibition of maize with a greater inhibitory effect on the growth of roots than of callus.

20. Abscisic acid showed a similar inhibitory effect on maize, wheat and sorghum tissues. In the presence of this chemical the number of roots in these three cereal cultures was reduced, the tissue consisting mostly of callus.

21. Cytidylic acid or adenine stimulated, and uracil inhibited the growth of maize. Wheat tissue growth was enhanced by several nucleotides added alone or in combination.

22. Maize and wheat tissues grew well over a wide pH range from 4.0 to 8.0.

23. Maize, wheat and rice showed characteristic sigmoid growth curves with a lag period, a logarithmic and a stationary phase but differed in the duration of the different growth phases.

24. When maize, wheat and sorghum tissues grown on agar, and consisting of varying proportions of callus and roots were transferred to liquid media of the same composition, and agitated on a shaker the tissue grew exclusively as a mass of roots. Rice cultures did not form roots under these conditions but grew only as callus.

25. The capacity of the roots of maize, wheat and sorghum to continue growing as roots in liquid medium with vigorous agitation was not lost even by repeated subculture for periods ranging from 1 to 3 years. The growth and maintenance of organized tissue under such conditions of vigorous agitation has not been described hitherto. These cultures in shake flasks represent root cultures of these cereals which are viable on subculture in vitro. This is the first report of continuous viable cultures of corn and sorghum roots.

26. The roots of maize, wheat and sorghum from liquid medium transferred back to agar medium were found to grow as a mixture of callus and roots. The growth of callus as roots in liquid cultures is therefore reversible. This reversibility effect was not lost even over a number of transfers from agar to liquid media and back.

27. The rate of growth of maize in shake flasks in 18 days was 3 to 4 times higher than on agar in 45 days. The growth of wheat and sorghum tissues in shake flasks was comparable to their growth on agar.

28. The medium required for the growth of maize roots in shake flasks was similar to that for growth on agar (modified Smith's basal medium, containing higher levels of the White's B vitamin solution, sucrose, edamin, naphthalene acetic acid and inositol.

29. The optimum edamin concentration for the growth of maize roots in liquid culture was 0.1 per cent.

30. Maize growth was stimulated by 0.01 ppm kinetin and by 0.1 ppm diphenyl urea whereas zeatin was inhibitory at levels of 0.05 ug per litre or more.

31. CCC, AMO-1618 and abscisic acid inhibited growth of maize roots in shake flasks.

32. The optimum 1-naphthalene acetic acid concentration was 5 ppm for maize; higher concentrations were inhibitory in liquid cultures.

33. Preliminary studies indicate that only thiamine and pyridoxine are required for the growth of maize roots in shake flasks. Optimum growth was obtained at an inositol concentration of 100 ppm.

34. Data on the uptake of phosphorus, organic nitrogen, sucrose and iron from the medium are presented for maize and wheat tissues in shake flasks.

35. Kinetin increased the uptake of phosphorus and organic nitrogen by maize even though growth was inhibited. The rate of phosphorus uptake by maize and wheat roots was higher as the inorganic phosphorus concentration of the medium was increased; it was relatively unaffected by the naphthalene acetic acid concentration.

36. The differentiation of maize, wheat and sorghum callus into roots was unaffected even after maintenance in vitro for 3 to 5 years. Differentiation into roots was favoured by decreasing the auxin concentration, by the addition of gibberellic acid, by increasing the period of incubation of the cultures and by vigorous agitation in liquid media. Shoot initiation was not observed under any of the conditions tested.

37. Maize and wheat tissue were grown on agar and in shake flasks on the same medium and a comparison was made of the callus tissue and roots in each case with respect to 15 enzymes. Both maize and wheat roots had a higher content of isocitrate dehydrogenase, malate dehydrogenase and glucose 6-phosphate dehydrogenase and a lower content of pyruvate kinase than the corresponding

callus tissues. The esterase content of maize roots was nearly 4 to 5 times higher than in callus.

38. Both callus and roots of maize and wheat contained significant amounts of only three sugars - sucrose, glucose and fructose. The amounts of these sugars in maize were nearly the same. In wheat callus and roots, fructose formed the major sugar and constituted over half the total carbohydrate content.

39. The number of cells per gram of dry tissue of maize and wheat was nearly the same between 18 and 40 days, whereas rice showed a slight increase in cell number upto the 40th day.

40. The RNA and DNA contents per gram dry tissue of maize, wheat and rice did not show very marked increases or decreases between the 18th and 40th day.

B I B L I O G R A P H Y

(Pages 220 - 236)

1. Adam, H. (1963) in Methods of Enzymatic Analysis, Acad. Press,N.Y., Ed. H. U. Bergmeyer, 573.

2. Addicott, F. T. and Lyon, Jessye L. (1969) Ann. Rev. Pl. Physiol. 20, 139.

3. Almextrand, A. (1949) Physiol. Plantarum, 2, 372.

4. Almestrand, A. (1950a) Physiol. Plantarum, 3, 205.

5. Almestrand, A. (1950b) Physiol. Plantarum, 3, 293.

6. Almestrand, A. (1951), Physiol. Plantarum, 4, 224.

7. Almestrand, A. (1957), Physiol. Plantarum, 10, 521.

8. Archibald, J. F. (1954), Nature, 173, 351.

9. Arya, H. C. (1964), J. Ind. Botan. Soc. XLIII, 229.

10. Arya, H. C., Hildebrandt, A. C., and Riker, A. J. (1962), Phyton, 19, 27.

11. Aspinal, D., Paleg, L. G., and Addicott, F. T. (1967) Austr. J. Biol. Sc. 20, 869.

12. Babcock, P. A., and Carew, D. P. (1962), Lloydia, 25, 209.

13. Baldev, B. (1962), Ann. Botany, 26, 173.

14. Ball, E. (1955), Annee Biol. 31, 80.

15. Barba, R. and Nickell, L. G. (1969), Planta, 89, 299.

16. Barker, G. R. and Hollinshead, J. A. (1964), Biochem. J. 93, 78.

17. Bellamy, A. R. and Bieleski, R. L. (1966), Austr. J. Biol. Sc. 19, 23.

18. Benveniste, P., Hirth, L. and Ourisson, G. (1964), Compt. Rend. 259, 2005.

19. Bergmann, L. (1958), Planta, 51, 70.

20. Bergmann, L. (1959), Naturwissenschaften, 46, 20.

21. Bergmann, L. (1960), J. Gen. Physiol. 43, 841.

22. Berthelot, A. (1934), Bull. Soc. chim. biol. Paris, 16, 1553.

23. Bernfeld, P. (1955) in Methods in Enzym. Vol. I, Ed. S.P. Colowick and N. O. Kaplan, Acad. Press, p. 149.

24. Bertrand, G., and Thomas, P. (1920), Pract. Biol. Chem., G. Bell & Sons, London, 61.

25. Bjorn, L. O. (1965), Physiol. Plantarum, 18, 1130.

26. Bjorn, L. O., Suzuki, Y. and Nilsson, J. (1963), Physiol. Plantarum, 16, 132.

27. Blaydes, D. F. (1966), Physiol. Plantarum, 19, 748.

28. Blumenfeld, A. and Gazit, S. (1970), Plant. Physiol. 45, 535.

29. Boder, G. B., Gorman, M., Johnson, I. S. and Simpson, P. J. (1964), Lloydia, 27, 328.

30. Bonner, J. (1940), Am. J. Botany, 27, 692.

31. Bonner, J. (1942c), Botan. Gaz. 103, 581.

32. Bonner, J. (1943), Bull. Torrey Botan. Club, 70, 184.

33. Bonner, J. and Bonner, H. (1948), Vitamins and Hormones, 6, 225.

34. Bonner, J. and Devirian, P. S. (1939), Am. J. Botany, 26, 661.

35. Braun, A. C. (1969) in Pl. Physiol. Vol. V B, Ed. F. C. Steward, Academic Press, p. 579.

36. Brian, P. W., Grove, J. F. and MacMillan, J. (1960), Prog. Chem. Org. Nat. Prod. 18, 350.

37. Brian, P. W., Hemming, H. G. and Lower, D. (1964), Ann. Botany. 28, 369.

38. Brown, E. G. and Short, K. C. (1969), Phytochem. 8, 1365.

39. Bruce, M. I., Zwar, J. A. and Kefford, N. P. (1965), Life Sci. 4, 461.

40. Bruce, M. I. and Zwar, J. A. (1966), Proc. Royal Soc. B, 165, 245.

41. Bryson, J. L. and Mitchell, T. J. (1951), Nature, 167, 864.

42. Bücker, T. and Hohorst, H. J. (1963) in Methods of Enzymatic Analysis Ed. H. U. Bergmeyer, Acad. Press, p. 246.

43. Buchner, S. A. and Staba, E. J. (1964), J. Pharm. Pharmacol. 16, 733.

44. Burstrom, H. (1941), Bot. Not. 1941, 310.

45. Burkholder, P. R. and Nickell, L. G. (1949), Botan. Gaz. 110, 426.

46. Burrows, W. J., Armstrong, D. J., Kameneck, M., Skoog, F., Bock, R. M., Hecht, S. M., Dammann, L. G., Leonard, L. J. and (1970), Biochemistry, 9, 1867.

47. Burton, K. (1956), Biochem. J. 62, 315.

48. Butenko, R. G. (1964) in Plant Tissue Culture and Plant Morphogenesis Ed. M. Kh. Chailakhyan, Moscow.

49. Caplin, S. M. (1963), Am. J. Botany, 50, 91.

50. Caplin, S. M. and Steward, F. C. (1948), Science, 108, 655.

51. Caplin, S. M. and Steward, F. C. (1949), Nature, 163, 920.

52. Caplin, S. M. and Steward, F. C. (1952), Ann. Botany, 16, 219.

53. Carew, D. P., Nylund, H. B., and Harris, A. L. (1964), Lloydia, 27, 322.

54. Carew, D. P. and Schwarting, A. E. (1958), Botan. Gaz. 119, 237.

55. Carew, D. P. and Schwarting, A. E. (1959), J. Am. Pharm. Assoc. Sci. 48, 499.

56. Carew, D. P. and Staba, E. J. (1965), Lloydia, 28, 1.

57. Carter, J. E. and Street, H. E. (1963), Physiol. Plantarum, 16, 347.

58. Carter, O., Yamada, Y. and Takahashi, E. (1967), Nature, 214, 1029.

59. Cathey, H. M. (1964), Ann. Rev. Plant Physiol. 15, 271.

60. Cathey, H. M. and Stuart, N. W. (1961), Botan. Gaz. 123, 51.

61. Chandra, N. and Hildebrandt, A. C. (1966), Science, 152, 789.

62. Chandra, N. and Hildebrandt, A. C. (1967), Virology, 31, 414.

63. Chang, C. K. and Carew, D. P. (1968), Lloydia, 31, 38.

64. Chen, H. R. and Galston, A. W. (1967), Physiol. Plantarum, 20, 533.

65. Derbyshire, E. and Street, H. E. (1964), Physiol. Plantarum, 17, 107.

66. Duhamet, L. (1951), C. R. Soc. Biol. Paris, 145, 1781.

67. Earle, E. D. and Torrey, J. G. (1963), Am. J. Botany, 50, 613.

68. Earle, E. D. and Torrey, J. G. (1965), Am. J. Botany, 52, 891.

69. Eriksson, T. (1965), Physiol. Plantarum, 18, 976.

70. Ferguson, J. D. (1963), Physiol. Plantarum, 16, 585.

71. Fergusson, J. D. (1967), Physiol. Plantarum, 20, 276.

72. Fiedler, H. (1936), Zeit. Bot. 30, 385.

73. Fiske, C. H. and Subba Row, Y. (1925), J. Biol. Chem. 66, 375.

74. Fleck, A. and Munro, H. N. (1962), Bioch. Biophy. Acta, 55, 571.

75. Fosket, D. E. and Roberts, L. W. (1964), Am. J. Bot. 51, 19.

76. Fujiwara, A. and Ojima, H. (1954), Tokoku J. Agr. Res. 5, 53.

77. Galston, A. W. (1948), Am. J. Botany, 35, 281.

78. Galston, A. W. and Davies, P. S. (1969), Science, 163, 1288.

79. Gamborg, O. L. and Eveleigh, D. E. (1968), Canad. J. Biochem. 46, 417.

80. Gautheret, R. J. (1934), Compt. Rend. 198, 2195.

81. Gautheret, R. J. (1937), Compt. Rend. 206, 572.

82. Gautheret, R. J. (1939), Compt. Rend. 208, 118.

83. Gautheret, R. J. (1941), Compt. Rend. Soc. Biol. 135, 875.

84. Gautheret, R. J. (1942) Manuel technique de culture des tissus vegetaux, Paris.

85. Gautheret, R. J. (1942a) Bull. Soc. Chem. Biob., Paris, 74, 13.

86. Gautheret, R. J. (1945), "Une voie nouvelle en biologie vegetale La culture des tissus", Gallimard, Paris.

87. Gautheret, R. J. (1948a) Compt. Rend. Soc. Biol. Paris, 142, 808.

88. Gautheret, R. J. (1955), Ann. Rev. Plant Physiol. 6, 433.

89. Gautheret, R. J. (1959) in "La Culture des tissus vegetaux", Ed. Masson et Cie., Paris.

90. Gautheret, R. J. (1966) in Cell Differentiation and Morphogenesis, North Holland Publishing Company, p. 55.

91. Gawron, O., Glaid, A. J. and Fondy, T. P. (1961), J. Am. Chem. Soc. 83, 3634.

92. Gentile, A. C. (1965) in "Tissue Culture", Ed. C. V. Ramakrishnan, W. Junk Publishers, p. 358.

93. Gibbons, G. S. B. and Wilkins, M. B. (1970), Nature, 226, 558.

94. Graebe, J. E. and Novelli, G. D. (1966a), Exptl. Cell Res. 41, 509.

95. Graebe, J. E. and Novelli, G. D. (1966b), Exptl. Cell Res. 41, 521.

96. Gudjonsdotter, S. and Burstrom, H. (1962), Physiol. Plantarum, 15, 498.

97. Haberlandt, G. (1902), Nat. Kl. Kais. Akad. Wiss. Wien, 111, 69.

98. Halperin, W. (1964), Science, 146, 408.

99. Halperin, W. (1966), Am. J. Botany, 53, 443.

100. Halperin, W. and Wetherell, D. F. (1965b), Nature, 205, 519.

101. Hannay, J. W., Fletcher, B. H. and Street, H. E. (1959), New Phytol. 58,142.

102. Harris, G. P. (1959), New Phytologist, 58, 330.

103. Heller, R. (1953), Ann. Sci. Nat., Bot. et Biol. Veg. II, 14, 1.

104. Henderson, J. H. M. (1954), Annee. Biol. 30, 329.

105. Henderson, J. H. M. (1958), Nature, 182, 880.

106. Henderson, J. H. M., Purrell, M. E. and Bonner, J. (1952), Am. J. Botany, 39, 467.

107. Hildebrandt, A. C. and Riker, A. J. (1949), Am. J. Botany, 36, 75.

108. Hildebrandt, A. C. and Riker, A. J. (1953), Am. J. Botany, 40, 66.

109. Hildebrandt, A. C., Riker, A. J. and Duggar, B. M. (1945), Am. J. Botany, 32, 357.

110. Hildebrandt, A. C., Riker, A. J. and Duggar, B. M. (1946), Am. J. Botany, 33, 591.

111. Hoagland, R. D. (1944) "Lectures on the Inorganic Nutrition of Plants", Chronica Botanica, Massachusetts.

112. Hohorst, H. J. (1963) in Methods of Enzymatic Analysis, Ed. H. U. Bergmeyer, Academic Press, N. Y. p. 328.

113. Hohorst, H. J. and Reim, M. (1963) in Methods of Enzymatic Analysis, Ed. H. U. Bergmeyer, Academic Press, N. Y. p. 335.

114. Horecker, B. L. and Smyrniotis, P. Z. (1951), J. Biol. Chem. 193, 371.

115. Horecker, B. L. and Smyrniotis, P. Z. (1953) Bioch. Biophy. Acta. 12, 98.

116. Huggins, C. and Lapides, J. (1947), J. Biol. Chem. 170, 465.

117. Jacquiot, C. C. R. (1950) C. R. Acad. Sci. Paris, 231, 1080.

118. Jablonski, J. R. and Skoog, F. (1954), Physiol. Plantarum, 7, 16.

119. Jagannathan, V., Singh, K. and Damodaran, M. (1956), Biochem. J. 63, 94.

120. Jagendorf, A. T., Bonner, D. M. and Naylor, A. W. (1952) . Bot. Gaz. 113, 334.

121. Juniper, B. E., Groves, S., Schachar, B. L. and Audus, L. J. (1966) Nature, 209, 93.

122. Kandler, O. (1950), Planta, 38, 564.

123. Kandler, O. (1952), Planta, 40, 346.

124. Kaufman, S. (1955), J. Biol. Chem. 216, 153.

125. Kefford, N. P. (1963), Science, 142, 1495.

126. Khan, A. A. (1967), Physiol. Plantarum, 20, 306.

127. Khan, A. A. and Faust, M. A. (1966), Nature, 211, 1215.

128. Khanna, Pushpa and Staba, E. J. (1967) in Seminar of Plant Cell and Organ Culture, Delhi, p. 41.

129. Klein, R. M. and Edsal, P. C. (1968) Phytomorphology, 18, 204.

130. Klein, R. M. and Manos, G. E. (1960), Ann. N.Y. Acad. Sci. 88, 416.

131. Knop, W. (1865), Landiv. Vers. Sta. 7, 93.

132. Kotte, W.(1922), Beitr. Allg. Bot. 2, 413.

133. Krikorian, A. D. and Berquam, D. L. (1969), Botan. Rev. 35, 59.

134. Krikorian, A. D. and Katz, G. M. (1968), Phytomorphology, 18, 207.

135. Krikorian, A. D. and Steward, F. C. (1969) in Plant Physiol. VB Ed. F. C. Steward, Academic Press.

136. Kubowitz, F. and Ott, P. (1943), Biochem. Zeit. 314, 94.

137. Kunitz, M. (1949), J. Gen. Physiol. 33, 349.

138. Lamport, D. T. A. (1964) Exptl. Cell Res. 33, 195.

139. Lance, C. (1958), Compt. Rend. Acad. Sci. 247, 959.

140. Lance, C. (1961), Compt. Rend. Acad. Sci. 252, 933.

141. Lance, C. (1962), Ph.D. Thesis, University of Paris, Masson and Co., Paris.

142. La Rue, C. D. (1947), Am. J. Botany, 34, 585.

143. La Rue, C. D. (1949), Am. J. Botany, 36, 798.

144. La Rue, C. D. and Smith, J. E. (1963), Science, 142, 1655.

145. Lavee, S. and Messer Glenda (1969), J. Exptl. Botany, 20, 604.

146. Letham, D. S. (1963a),New Zealand J. Botany, 1, 336.

147. Letham, D. S. (1966) Phytochem. 5, 269.

148. Letham, D. S., Shannon, J. S. and Mac Donald, I. R. (1964), Proc. Chem. Soc. p. 230.

149. Lin, M. and Staba, E. J. (1961), Lloydia, 24, 139.

150. Linsmaier, E. M. and Skoog, F. (1965), Physiol. Plantarum, 18, 100.

151. Loewenberg, J. R. and Skoog, F. (1952), Physiol. Plantarum, 5, 33.

152. Loo Shih-Wei (1945), Am. J. Botany, 32, 13.

153. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951), J. Biol. Chem. 193, 265.

154. Maeda, E. (1968), Proc. Crop. Sci. Soc. Japan, 37, 51.

155. Maheswari, S. C. and Gupta, G. R. P. (1967), Life Sciences, 6, 2459.

156. Maheswari, S. C. and Prakash, R. (1967), Life Sciences, 6, 2453.

157. Maretzki, A. and Nickell, L. G. (1967), Abst. VII, Intern.Cong.Biochem.

158. Maretzki, A., and Nickell, L. G., and Thom, M. (1969), Physiol. Plantarum, 22, 827.

159. Marth, P. C., Preston, W. H. and Mitchell, J. W. (1953), Botan. Gaz. 115, 200.

160. Mascarenhas, A. F. (1965), M.Sc. Thesis,"Biochemical Studies on Plant Tissue Cultures. Studies on Vigna catjang cells".

161. Mascarenhas, A. F., Hendre, R. R., Seetha Rama Rao, B. and Jagannathan, V. (1969), Ind.J.Exptl.Biol. 7, 65.

162. Mascarenhas, A. F., Sayagaver, B. M., and Jagannathan, V. (1965) in Tissue Culture, Ed. C.V.Ramakrishnan, W.Junk Publishers,283.

163. Masteller, V. and Holden, D. J. (1970), Pl. Physiol. 45, 362.

164. Mathes, M. C. (1963), Science, 140, 1101.

165. Mathes, M. C. (1964), Phyton, 21, 137.

166. Mathes, M. C. (1967), Canad. J. Botany, 45, 2195.

167. McClary, J. E. (1940), Proc. Natl. Acad. Sci. 26, 581.

168. Milborrow, B. V. (1966), Planta, 70, 155.

169. Miller, C. O. (1961), Ann. Rev. Pl. Physiol. 12, 395.

170. Miller, C. O. (1961a), Proc. Natl. Acad. Sci. 47, 170.

171. Miller, C. O. (1963), in Modern Methods of Pl. Analysis. Ed. Linskens, H. F. and Tracey, M. V. Vol. 6, 194.

172. Miller, C. O. (1965), Proc. Natl. Acad. Sci. 54, 1052.

173. Miller, C. O. (1965), Pl. Physiol. (Suppl.), 40, lxxviii.

174. Miller, C. O. and Skoog, F. (1953), Am. J. Botany, 40, 768.

175. Miller, C. O. and Witham, F. H. (1964) in Regul. natur. de la croissance vegetale, Ed. Nitsch, J. P. Paris, 1.

176. Miller, C. O., Skoog, F., Okumura, F. S., von Saltza, M. H. and Strong, F. M. (1956), J. Am. Chem. Soc. 78, 1375.

177. Miller, R. A., Shyluck, J. P., Gamborg, O. L., and Kirkpatrick, J.W. (1968), Science, 159, 540.

178. Mishra, M. D. (1967) in Seminar Plant, Cell, Tissue and Organ Culture, Ed. Johri, B. M., Delhi, 57.

179. Mitra, J., Mapes, M. O. and Steward, F. C. (1960), Am. J. Botany, 47, 357.

180. Mitra, J. and Steward, F. C. (1961), Am. J. Botany, 48, 358.

181. Mohan Ram, H. Y. and Steward, F. C. (1964), Canad. J. Botany, 42, 1559.

182. Morel, G. (1944), Campt. Rend. 218. 50.

183. Morel, G. (1946), Compt. Rend. 223, 166.

184. Morel, G. (1948), Ann. Epiphyties, N. S. 14, 123.

185. Morel, G. (1960), Am. Orchid Soc. Bull. 29, 495.

186. Morel, G. and Martin, C. (1952), C.R. Hebd. Seanc. Acad. Sci. (Paris), 235, 1324.

187. Morel, G. and Wetmore, R. H. (1951), Am. J. Botany, 38, 138.

187a. Muir, W. H. (1965) in Proc. Intern. Conf. Pl. Tissue Cult. Ed. White, P. R. and Grove, A. R., Mc Cutchan Publ. Corp. 485.

188. Muir, W. H., Hildebrandt, A. C. and Riker, A. J. (1954) Science, 119, 877.

189. Mullin, M. (1970), Austral. J. Biol. Sci. 23, 473.

190. Murashige, T. (1961), Science, 134, 280.

191. Murashige, T. (1964), Physiol. Plantarum, 17, 636.

192. Murashige, T. and Nakano, R. (1966), J. Heredity, 57, 114.

193. Murashige, T. and Skoog, F. (1962), Physiol. Plantarum, 15, 473.

194. Nickell, L. G. (1950a), Nature, 166, 351.

195. Nickell, L. G. (1950b), Bot. Gaz. 112, 225.

196. Nickell, L. G. (1956), Proc. Natl. Acad. Sci. 42, 848.

197. Nickell, L. G. (1964), Hawaiian Planter's Record, 57, 223.

198. Nickell, L. G. (1967) in Proc. 12th Congr. Int. Soc. Sugarcane Techn. Puerto Rico, 887.

199. Nickell, L. G. and Burkholder, P. R. (1950), Am. J. Botany, 37, 538.

200. Nickell, L. G. and Maretzki, A. (1969), Physiol. Plantarum, 22, 117.

201. Nickell, L. G. and Maretzki, A. (1970), Pl. Cell Physiol. 11, 183.

202. Nickell, L. G. and Tulecke, W. (1959) in Symposium on "Mass propagation of Cells", Atlantic City, September 15.

203. Nickell, L. G. and Tulecke, W. (1959a), Bot. Gaz. 120, 245.

204. Nickell, L. G. and Tulecke, W. (1960), J. Bioch. & Microb. Tech. and Engin. II, 287.

205. Niizeki, H., and Oono, K. (1968), Proc. Japan Acad. 44, 554.

206. Nishi, Toyoyuki; Yamada, Y., and Takahashi, E. (1968), Nature, 219, 508.

207. Nitsch, J. P. (1951), Am. J. Bot. 38, 566.

208. Nitsch, J. P. (1963) in Pl. Tissue and Organ Culture - A symposium - Ed. Maheswari, P. and Rangaswamy, N. S., Catholic Press, India, 144.

209. Nitsch, J. P. and Nitsch, C. (1957), Am. J. Botany, 44, 555.

210. Nitsch, J. P., Nitsch, C. and Hamon, S. (1969), C.R. Acad.Sc. Paris, 269, 1275.

211. Nitsch, J. P. and Nitsch, C. (1969), Science, 163, 85.

212. Nobecourt, P. (1939), Compt. Rend. Soc. Biol. 130, 1270.

213. Norstog, K. (1956), Bot. Gaz. 117, 253.

214. Norstog, K., Wall, E. Wendell, and Howland, G. P. (1969), Bot. Gaz. 130, 83.

215. Ojima, K. and Fujiwara, A. (1959), Tohoku J. Agri. Res. 10, 111.

216. Ojima, K. and Fujiwara, A. (1960), Tohoku J. Agri. Res. 11, 201.

217. Ojima, K. and Fujiwara, A. (1962), Tohoku J. Agri. Res. 13, 69.

218. Overbeek, J. van., Conklin, M. E. and Blakeslee, A. F. (1941), Science, 94, 350.

219. Overbeek, J. van., Tukey, H. B., Went, F. W. and Muir, R. M. (1954), Pl. Physiol. 29, 307.

220. Overbeek, J. van. (1968) in Plant Growth Regulators, S.C.I. Monograph, No. 31, p. 181.

221. Paleg, L. G. (1965) Ann. Rev. Pl. Physiol. 16, 291.

222. Parekh, L. J., Antony, A. and Ramakrishnan, C. V. (1969), Phytochem. 8, 1167.

223. Paris, D., Duhamet, L. and Goris, A. (1954), C.R. Soc. Biol. Paris, 148, 296.

223a. Partridge, S. M. (1948), Biochem. J. 42, 238.

224. Petru, E. (1966) in Proc. of a symp. Differentiation of Apical meristems and some problems of ecological regulation of development of plants. Published Czechoslovak Acad. Sciences, 79.

225. Pilet, P. E. and Bonhote, J. (1962), Ber. Schweiz. Botan. Ges. 72, 5.

226. Plaisted, P. H. (1958) Contr. Boyce Thompson Inst. 19, 231.

227. Pollard, J. K., Shantz, E. M. and Steward, F. C. (1961), PI. Physiol.36,492.

228. Racker, E., Colowick, S. P. and Kaplan, N. O. (1957) in Methods in Enzymology, Academic Press, N. Y. III, 295.

229. Raghavan, V. and Torrey, J. G. (1964), Pl. Physiol. 39, 691.

230. Ranganathan, B., Mascarenhas, A. F., Sayagaver, B. M. and Jagannathan, V. (1963), in Pl. Tissue & Organ Culture - A symposium, Ed. Maheshwari, P. & Rangaswamy, N. S. Catholic Press, India, 108.

231. Rao, A. N. (1963) in Pl. Tissue & Organ Culture - A symposium
Ed. Maheshwari, P. & Rangaswamy, N. S.
Catholic Press, India, 332.

232. Rao, C. N. and Srinivasulu, K. (1966), Curr. Sci. 35, 490.

233. Ray Chaudhuri, S. P. (1966) in "Advances in Virus Research", 12, 175.

234. Reinert, J. and White, P. R. (1956), Physiol. Plantarum, 9, 177.

235. Riker, A. J. and Gutsche, A. E. (1948). Am. J. Botany, 35, 227.

236. Risser, P. G. and White, P. R. (1964), Physiol. Plantarum, 17, 620.

237. Robb, S. M. (1957), J. Exptl. Botany, 8, 348.

238. Roberts, E. H. and Street, H. E. (1955), Physiol. Plantarum, 8, 238.

239. Robbins, W. J. (1922), Bot. Gaz. 73, 376.

240. Robbins, W. J. (1951) in Plant Growth Substances, Ed. F.Skoog, 463.

241. Robbins, W. J. and Schmidt, M. B. (1939a), Proc.Natl.Acad.Sci. 25, 1.

242. Robbins, W. J. and Schmidt, M. B. (1939b), Am. J. Botany, 26, 149.

243. Robbins, W. J. and Schmidt, M. B. (1939c), Bull.Torrey Bot. Club.
66, 193.

244. Roth, J. S. and Milstein, S. W. (1962), J. Biol. Chem. 196, 489.

245. Sandell, E. B. (1944), Color detm. of trace metals, Interscience
Publishers, N. Y.

246. Sanders, M. E. and Burkholder, P. R. (1948), Proc. Natl. Acad. Sci.
34, 516.

247. Sargent, J. A. and Skoog, F. (1960), Pl. Physiol. 35, 934.

248. Sargent, J. A. and Skoog, F. (1961), Physiol. Plantarum, 14, 504.

249. Schneider, G., Erdmann, D., Lust, S., Mohr, G. and Weithammer, K.
(1965), Nature, 208, 1013.

250. Scott, E. G., Carter, J. E. and Street, H. E. (1961),
Physiol. Plantarum, 14, 725.

251. Seitz, E. W. and Hochster, R. M. (1964), Canad. J. Botany, 42, 999.

252. Sexton, R. and Sutcliffe, J. F. (1969), Ann. Botany, 33, 407.

253. Shantz, E. M. and Steward, F. C. (1952), J. Am. Chem. Soc. 74, 6133.

254. Shantz, E. M. and Steward, F. C. (1955), J. Am. Chem. Soc. 77, 6351.

255. Shantz, E. M., Mears, K., and Steward, F. C. (1958)
Pl. Physiol. (Suppl.), 33, XVI.

256. Sheat, D. G., Fletcher, B. H. and Street, H. E. (1959)
New Phytol. 58, 128.

257. Sheridan, W. F. (1968), Planta, 82, 189.

258. Shimada, T., Sasakuma, T. and Tsunewaki, K. (1969)
Canad. J. Genet. Cytol. XI, 294.

259. Short, K. C., Brown, E. G., and Street, H. E. (1969),
J. Exptl. Botany, 20, 579.

260. Simpkins, I., Collin, H. A., and Street, H. E. (1970)
Physiol. Plantarum, 23, 385.

261. Skoog, F. (1944), Am. J. Botany, 13, 19.

262. Skoog, F., and Miller, C. O. (1957), Symp. Soc. Exptl. Biol. 11, 118.

263. Skoog, F., and Tsui, C. (1948), Am. J. Botany, 35, 782.

264. Smith, C. W. (1965), Nature, 207, 780.

265. Smith, W. C. (1967) in Methods in Virology, Ed. K. Maramorosch
and H. Koprowski, Academic Press, Vol. I, 553.

266. Speake, T., McCloskey, P., Smith, W. K., Scott, T. A. and Hussey, H.
(1964), Nature, 201. 614.

267. Srivastava, S. K., Maini, S. B., and Ramakrishnan, C. V. (1969)
Phytochem. 8, 1155.

268. Staba, E. J. (1962), J. Pharmaceut. Sci. 51, 249.

269. Staba, E. J. and Lamba, S. S. (1963), Lloydia, 26, 29.

270. Staba, E. J., and Laursen, P. and Buchner, S. A. (1965) in
Conference on medicinal plant tissue cultures, 1963,
Pennsylvania, Am. Inst. Biol. Sci., Washington, D. C.

271. Steeves, T. A., and Sussex, I. M. (1952), Nature, 170, 672.

272. Steinhart, C. E., Standifer, L. G., and Skoog, F. (1961),
Am. J. Botany, 48, 465.

273. Sternheimer, E. P. (1954), Bull. Torrey Bot. Club, 81, 111.

274. Steward, F. C. (1963) in Plant Tissue and Organ Culture - A symposium, Ed. P. Maheshwari and N. S. Rangaswamy, Catholic Press, India, 1.

275. Steward, F. C. (1970), Proc. Royal Soc. (Lond.) B, 175, 1.

276. Steward, F. C. and Caplin, S. M. (1951), Science, 113, 518.

277. Steward, F. C. and Mohan Ram, H. Y. (1961), Adv. Morphogenesis, 1, 189.

278. Steward, F. C., Mapes, M. O. and Mears, K. (1958 a), Am. J. Botany, 45, 705.

279. Steward, F. C., Mapes, M. O. and Smith, J. (1958), Am. J. Botany, 45, 693.

280. Steward, F. C., Bidwell, R. G. S. and Yemm, E. W. (1958), J. Exptl. Botany, 9, 11.

281. Steward, F. C., Hulme, A. C., Freiberg, S. R., Hegarty, M. P., Pollard, J. K., Rabson, R., and Barr, R. A. (1960), Ann. Botany, 24, 83.

282. Steward, F. C., Shantz, E. M., Pollard, J. K., Mapes, M. O., and Mitra, J. (1961), in Molecular and Cellular Structure, Ronald Press, N. Y. 193.

283. Steward, F. C., Blakely, L. M., Kent, A. E. and Mapes, M. O. (1963), in 16th Brookhaven Nat. Lab. Symp. Biol. 83.

284. Steward, F. C., Mapes, M. O., Kent, A. E. and Holston, R. D. (1964), Science, 143, 20.

285. Steward, F. C., Kent, A. E. and Mapes, M. O. (1966), Current Topics Develop. Biol. 1, 113.

286. Steward, F. C., Kent, A. E. and Mapes, M. O. (1967), Ann. N. Y. Acad. Sci. 144, 326.

287. Steward, F. C., Mapes, M. O., and Ammirato, P. V. (1969) in Pl. Physiol. V B, Ed. F.C. Steward, Academic Press, 329.

288. Straus, J. (1954), Am. J. Botany, 41, 833.

289. Straus, J. (1958), Science, 128, 537.

290. Straus, J. (1959), Pl. Physiol. 34, 536.

291. Straus, J. (1960), Pl. Physiol. 35, 645.

292. Straus, J. (1960), Am. J. Botany, 47, 641.

293. Straus, J. (1962), Pl. Physiol. 37, 324.

294. Straus, J. and Campbell, W. A. (1963), Life Sciences, 1, 60.

295. Straus, J. and La Rue, C. D. (1954), Am. J. Botany, 41, 687.

296. Straus, J. and Gerding, R. K. (1963), Pl. Physiol. 38, 621.

297. Street, H. E. (1959) in Encyclopaedia of Pl. Physiol.
Ed. W. Ruhland, II, Springer-Verlag, Berlin, 153.

298. Street, H. E. (1966) in Cells and Tissues in Culture, III,
Ed. E. N. Willmer, 533, 604, 631.

299. Street, H. E. (1969) in Pl. Physiol. V B, Ed. F. C. Steward,
Academic Press, 3, 145.

300. Street, H. E., Mc Gonagle, M. P., and Mc Gregor, S. M. (1952)
Physiol. Plantarum, 5, 248.

301. Street, H. E., Carter, J. E., Scott, E. G. and Sutton, D. (1961)
Physiol. Plantarum, 14, 621.

302. Street, H. E. and Henshaw, G. G. (1966) in Cells and Tissues in
Culture, III. Ed. E. N. Willmer, 459.

303. Strong, F. M. (1958) in Topics in Microbial Chemistry,
J. Wiley & Sons, 127.

304. Stuart, R. and Street, H. E. (1969), J. Exptl. Botany, 20, 556.

305. Sunderland, N. and Wicks, F. M. (1969), Nature, 224, 1227.

306. Sutton, D., Scott, E. G. and Street, H. E. (1961), Physiol. Plantarum,
14, 712.

307. Syono Kunihiko and Furuya, T. (1968), Pl. & Cell Physiol. 9, 103.

308. Talbot, B. and Street, H. E. (1968), Physiol. Plantarum, 21, 800.

309. Talbot, B. and Street, H. E. (1968), Physiol. Plantarum, 21, 971.

310. Tamaoki, T. and Ullstrup, A. J. (1958), Bull. Torrey Bot. Club, 85, 260.

311. Tamaoki, T., Hildebrandt, A. C., Riker, A. J., Burris, R. H. and
Hagihara, B. (1959), Nature, 184, 1491.

312. Tamaoki, T., Hildebrandt, A. C., Burris, R. H., Riker, A. J. and
Hagihara, B. (1960), Pl. Physiol. 35, 942.

313. Tamaoki, T., Hildebrandt, A. C., Burris, R. H. and Riker, A. J. (1961), Pl. Physiol. 36, 347.

314. Thomas, T. H., Wareing, P. F. and Robinson, P. M. (1965), Nature, 205, 1250.

315. Tiwari, M. M. and Arya, H. C. (1967), Ind. Phytopath. XX, 356.

316. Torrey, J. G. (1957), Proc. Nat. Acad. Sc. 43, 887.

317. Torrey, J. G. (1966), Adv. in Morphogen. 5, 39.

318. Torrey, J. G. (1967), Physiol. Plantarum, 20, 265.

319. Torrey, J. G. and Nickell, L. G. (1969), Science, 166, 1068.

320. Torrey, J. G. and Reinert, J. (1961), Pl. Physiol. 36, 483.

321. Torrey, J. G. and Shigemura, Y. (1957), Am. J. Botany, 44, 334.

322a. Trione, E. J. (1964), Phytopath. 54, 592.

322. Trione, E. J., Jones, L. E., and Metzger, R. J. (1962), Pl. Physiol. 37, 111.

323. Trione, E. J., Jones, L. E. and Metzger, R. J. (1968) Am. J. Botany, 55, 529.

324. Tulecke, W. (1953), Science, 117, 599.

325. Tulecke, W. (1957), Am. J. Botany, 44, 602.

326. Tulecke, W. (1963), Am. Biol. Teacher, 25, 90.

327. Tulecke, W., and Nickell, L. G. (1959), Science, 130, 863.

328. Tulecke, W., and Nickell, L. G. (1960), Trans. N.Y.Acad. Sci. 22, 196.

329. Tulecke, W., Weinstein, L. H., Rutner, A., and Laurencot, H. J. (1961), Contr. Boyce Thompson Inst. 21, 115.

330. Tulecke, W., Taggart, R., and Colavito, L. (1965), Contrib. Boyce Thompson Inst. 23, 33.

331. Tung, Heng Fong and Raghavan, V. (1968), Ann. Botany, 32, 509.

332. Utter, M. F. and Kurahashi, K. (1954), J.Biol.Chem. 207, 787.

333. Vacin, E. and Went, F. (1949), Bot. Gaz. 110, 605.

334. Vasil, I. K., Hildebrandt, A. C. and Riker, A. J. (1964), Science, 146, 76.

335. Vasil, I. K., and Hildebrandt, A. C. (1966a), Am. J. Botany, 53, 860.

336. Vasil, I. K., and Hildebrandt, A. C. (1966b), Am. J. Botany, 53, 869.

337. Vasil, V., and Hildebrandt, A. C. (1967), Planta, 76, 139.

338. Veldstra, H. (1962) in Comprehensive Biochemistry, Ed. M. Florkin and E. H. Stotz, Elsevier Press, Amsterdam, 2, 127.

339. Verma, D. P. S., and van Huystee, R. B. (1970), Canad. J. Botany, 48, 429.

340. Wang, C. J. and Staba, E. J. (1963), J. Pharmaceut. Sci. 52, 1058.

341. Warburg, O., Christian, W., and Griese, A. (1935), Biochem. Zeit. 282, 157.

342. Warburg, O., and Christian, W. (1941), Biochem. Zeit. 310, 384.

343. Webster, J. M. (1966), Nature, 212, 1472.

344. Went, F. W. and Thimann, K. V. (1938), in Phytohormones, MacMillan Press.

345. Weinstein, L. H., Nickell, L. G., Laurencot, H. J. and Tulecke, W. (1959), Contrib. Boyce Thompson Inst. 20, 239.

346. Weinstein, L. H., Tulecke, W., Nickell, L. G., and Laurencot, H. J. (1962), Contrib. Boyce Thompson Inst. 21, 371.

347. Wetmore, R. H. and Rier, J. P. (1963), Am. J. Botany, 50, 418.

348. White, P. R. (1932b), Pl. Physiol. 19, 97.

349. White, P. R. (1934), Pl. Physiol. 9, 585.

350. White, P. R. (1937a), Pl. Physiol. 12, 803.

351. White, P. R. (1939), Bull. Torrey Botan. Club, 66, 505.

352. White, P. R. (1943a), in Handbook of Plant Tissue Culture, J. Cattell, Lancaster.

353. White, P. R. (1943), Am. J. Botany, 30, 33.

354. White, P. R. (1954),"The Cultivation of Animal and Plant Cells", 1st edition, Ronald Press.

355. White, P. R. (1963), in The Cultivation of animal and plant cells, 2nd edition, Ronald Press.

356. White, P. R. (1967), in Abstracts of Seminar on Plant Tissue and Organ Culture, Ed. B. M. Johri, Delhi, 12.

357. White, P. R. and Braun, A. C. (1942), Cancer Res. 2, 597.

358. White, P. R. and Risser, P. G. (1964), Physiol. Plantarum, 17, 600.

359. White, P. R. and Gilbey, S. N. (1966), Physiol. Plantarum, 19, 177.

360. Wilmar, C. and Hellendoorn, M. (1968), Nature, 217, 369.

361. Wolter, K. E. and Skoog, F. (1966), Am. J. Botany, 53, 263.

362. Wood, H. N. and Braun, A. C. (1961), Proc. Nat. Acad. Sci. Wash. 47, 1907.

363. Yamada, Y., Tanaka, K. and Takahashi, E. (1967), Proc. Japan Acad, 43, 156.

364. Yamada, Y., Nishi, T., Yasuda, T. and Takahashi, E. (1968), in Advances in germ free research and Gnotobiology, Ed. Miyagawa and Luckey, Iliffe Books Ltd., London, 377.

365. Yatazawa, M., Furuhashi, K. and Shimizu, M. (1967), Pl. and Cell Physiol. 8, 363.

366. Yatazawa, M., Katsuhesa, F. and Teruji, S. (1967), Nippon Dojo-Hiryogaku Zasshi, 38, 305.

367. Yatazawa, M., Kurihara, N. and Furuhashi, K. (1968), Soil Sc. Plant Nut. (Tokyo), 14, 147.

368. Yatazawa, M., Furuhashi, K., Kurihara, N., and Ohnishi, Y. (1968), Soil Sc. Plant Nut. (Tokyo), 14, 85.

369. Ziv, M., Halevy, A. H. and Shilo, R. (1970), Ann. Botany, 34, 671.