BIOCHEMICAL STUDIES ON PLANT TISSUE CULTURES: Studies on *Vigna catiang* calls

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CONTENTS

LIST OF ABBREVIATIONS

The following abbreviations have been used in the tables, plates & figures.

YE	Yeast extract
ME	Malt extract
CM	Coconut milk
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	Naphthalene acetic acid
IAA	Indole acetic acid
2-BTOA	2,Benzo thiozolyl oxyacetic acid
WB	White's basal i.e., White's 'A' + White's 'B'

INTRODUCTION

INTRODUCTION

Plant tissue culture can be described in the words of White (1959) as "the method or methods by which the elementary units, that is, the cells are removed from the complex in which one normally finds them, that is, the body, and are then provided with suitable substitute environments which shall ideally in no way impair their normal functional capacities. They are thus made available for direct observation and experiment".

The first observations were made as early as 1665 by Robert Hooke on the cellular organization of plants. On examining charcoal, cork and other plant tissues under the microscope he found in them small honey-comb like cavities which he called "cells". These observations however were made on dead cells. A hundred and fifty years later Robert Brown in 1823 observed that cells, especially of plants, contain nuclei and that normally there is a single nucleus in each cell, and Dujardin in 1835 noted that the semi-fluid substance which commonly covers the cellular skeleton in the living parts of plants and animals has also a very important role to play. This semi-fluid substance he called the "sarcode" which is now known as the "protoplasm". In 1838 Schleiden and Schwann (1839) formulated jointly the cell theory based

on earlier observations of the "cell" by Hooke, of the "nucleus" of Brown and of the "sarcode" by Dujardin. They concluded that all plants and animals are made of cells and that the cell is the primary agent of organization and that each cell leads a double life, one pertaining to its own self, and another, a social one in respect to other cells of the organism. The first aspect of the theory, namely that the cell was a universal structural unit of all living creatures, has been accepted but the idea that each cell is an individual capable of autonomous existence and that an animal or a plant is no more than a society of co-operating cells has not received general acceptance. The latter aspect of the theory has been severely criticised by Sachs (1880) and others on the grounds that on account of the high degree of physiological differentiation among the various tissues and organs, the cell cannot be regarded as an independent unit but only as an integral part of a higher individual organisation.

However the only way for studying cellular autonomy and inter-relations would be by taking the organs, tissues and cells of a plant or animal and determining how they behave as isolated units. This idea of isolating cells and growing them for the purpose of studying cellular autonomy was put forward by Haberlandt (1902) who made the first recorded studies on the cultivation of isolated plant

cells, thus laying the foundations of the technique which is now known as "tissue culture". Using mature palisade cells from the leaves of Lamium purpureum and hair cells of Tradescantia and Pulmonaria he showed that such cells could survive as long as fifteen days in the mineral solution of Knop containing 1 to 5 per cent sucrose. Although such cells enlarged and developed starch granules in their chloroplasts he did not observe them divide. In 1917 Bobilioff-Preisser maintained leaf parenchyma cells alive for as long as a month on agar supplemented with various nutrients. Protoplasmic streaming was observed and the nucleus was found to be in a state of almost continuous movement, but again no signs of cell division were noticed. Essentially the same results were obtained also by Czeck (1926), Berger (1926) and Kommer (1928).

We now know that Haberlandt and other early investigators were unsuccessful probably because they chose plant materials which were unsuitable or difficult to culture and because the nutritional and physico-chemical requirements for growth were not understood clearly at that time.

In 1922, Kotte, a student of Haberlandt, and Robbins succeeded independently in growing excised plant roots for some weeks in a nutrient solution, but when these roots were re-excised and subcultured on fresh media, their growth

rate diminished and the roots died, indicating perhaps a deficiency of some substance in the medium.

In the related field of animal tissue culture, the first cultures were prepared in 1907 by Harrison using frog nerve tissue in a hanging drop of frog lymph. Among the pioneers in this field are Burrows and Alexis Carrel who developed the use of embryo juice as a growth promoting nutrient for animal tissues. Since then work in this field has made rapid progress both in the technique of handling the tissues and in its applications.

In 1933 Gautheret showed that cells of the root cap of Lupinus albus could be completely isolated from the root, and that such cells could increase in volume in about 20 days. This enlargement was increased by the presence of a root in the vicinity and also by a concentration of about one microgram per millilitre of indoleacetic acid. These isolated cells however did not survive after the initial period of growth.

It was White (1934a) who was able to determine the cause of the diminishing growth rate of isolated root cultures. He found that besides sucrose and the mineral salt medium of Uspenski and Uspenskaia (1925) excised tomato roots required niacin, pyridoxine and thiamine for continuous growth. Thus the first tissue or organ culture

of plant origin, which could be maintained over several passages, was successfully established. About the same time Gautheret (1934) carried out studies on cambial tissues of willow and poplar. These studies were continued further and in 1939 Gautheret, Nobecourt (1937, 1939a) and White (1939a) simultaneously and independently published data on the successful culture of cambial tissues of tomato and carrot over prolonged periods of time. Subsequent to these pioneering studies exciting advances have been made in the cultivation of a variety of plant materials in vitro and this new technique of "tissue culture " or "cell culture" has been extensively used for studies on the metabolism, biochemistry, nutrition, cell differentiation and other fields of plant physiology.

Media for tissue cultures

There have been a number of different media developed for the culture of isolated tissues of higher plants. Basically most media contain inorganic salts including nitrate as a source of nitrogen, sucrose for carbon, vitamins, mainly pyridoxine, nicotinic acid and thiamine, and distilled water. In addition some tissues also require supplements of complex extracts and growth factors. Agar may also be added in sufficient amount to give the tissue a firm support for aeration at the surface of the medium. Media prepared according to the formulae of White (1942), Gautheret (1942a), Murashige (1962), Hildebrandt (1946) are some of the more

common media which are generally used.

Types of cultures:

Extensive studies have been made of the growth of cells from higher plants and these cultures may be classified for convenience into: 1) Organ Cultures, 2) Callus cultures, 3) Tumor cultures, 4) Single cell cultures, 5) Submerged cultures.

1) Organ cultures:

The first successful organ culture as indicated earlier, was obtained by White (1934) with excised tomato roots. These roots have been growing with undiminished vigour in vitro for over thirty years with regular subcultures. Though practically every organ of the plant has been cultured till maturity, it has not been possible except with excised roots (Street et al. 1952; Ferguson, 1963; David & Street, 1961) to maintain the respective organs in culture for prolonged periods by subculture. Sussex and Steeves (1953) showed that excised fern leaves could be grown to maturity in sterile culture. Raghavan and Jacobs (1961) observed that apical buds of the short day plant Perilla frutescens produced normal flowers when cultured on syathetic media. Similarly Tapfer et al (1963) grew floral buds of Aquilegia at different stages of development on various agar media and found that when indoleacetic acid, gibberellic acid and kinetin were added to the basic medium, the developmental limits of buds were extended at nearly all stages and

decidedly improved the continued development of carpels. On this medium the buds grew from the early stages to about the size of flowers. If a flower is excised and properly surface sterilised and planted on a simple medium solidified with agar, or on a filter paper stand dipping in liquid medium, a whole ovary can develop similar to that which is formed when the flower is left attached to the plant. This was first observed by Nitsch in 1951 who obtained the tomato fruit in culture from the excised flowers. In 1963 Johri and Bajaj studied the nutritional requirements of Dendrophthoe falcata embryos and the formation of accessory embryos in vitro. Recently Maheshwari and Kantha (1965) have reported on the technique of fertilization of ovules in test tubes following the culturing of young ovules (excised before pollination) and mature pollen grains on the same nutrient medium.

2) Callus cultures:

The above examples deal with parts of the plant which have been cultured as independent organs. However the excised portion of the plant that is cultured on the medium can also form a disorganised mass of cells called callus and these callus cells can be maintained in vitro over a period of many years in several cases without differentiation. In some, though not in all instances callus cells apparently have lost most of their ability for morphogenesis except that of cell division. Innumerable callus cultures have now

been obtained from various parts of the living plant since the pioneering work of Gautheret (1939), Nobecourt (1939a) and White (1939a).

Roots:

In 1944 Skoog obtained callus on cultured roots from hybrid tobacco tissues in culture and in 1952 Jagendorf working with cabbage seedling roots obtained an atypical type of callus growth when parachlorophenoxyacetic acid (POA) was incorporated in the medium. Tryon in 1955 obtained a similar type of growth when roots of Nicotiana affinis seedlings were cultured in vitro on a medium containing yeast extract and malt extract. In 1957 Torrey and Shigemura obtained root callus tissue from excised root tips of germinated pea seeds, variety Alaska. This clone of pea root callus tissue was shown to require both yeast extract and auxin for increase in weight and was also shown to be capable of developing as roots in auxin-free media. Carrot root tissue has also been cultured extensively in vitro as unorganised callus masses by a number of workers in this field, most notably by Steward and his coworkers. An exciting development in this field is the demonstration by Steward (1963) of the growth of complete plants from carrot phloem cells. This reproductive capacity of a non-reproductive part of the plant shows that under suitable conditions the cells grown in vitro are totipotent and can undergo differentiation and give rise to whole plants.

Stem:

Callus cultures have been obtained from the cambial regions of the stem of a number of mature trees or even from the stem portions of seedlings. Ball in 1950 obtained callus from the woody stem of redwood burl (Sequoia sempervirens). Loewenberg and Skoog (1952) obtained callus from seedlings of four varieties of pine on a medium containing kinetin and malt extract. Jacquiot (1955) obtained callus tissue from the secondary cambium of elm (Ulmus campestris), birch (Betula verrucosa) and basswood (Zilia parvifolia) on standard media. This tissue was found to show varying degrees of differentiation from completely undifferentiated tissue to more or less normal roots and shoots. In 1960 Pelet et al obtained in vitro cultures from grape, elm, poplar and willow trees on White's nutrient medium with coconut milk and various growth factors. Callus tissues have also been obtained from the stems of a very large variety of different plants by a number of different workers.

Leaves:

In 1959 Weinstein et al obtained callus cultures from the leaves of Agave tourneyana seedlings grown on White's basal medium containing coconut milk and 2,4-dichlorophenoxy-acetic acid. Working with leaves of Kalanchoe pinnata Mohan Ram and Wadhi (1965) were able to obtain callus cultures from a section of the notches cultured in vitro and Vasil et al (1964a) were successful in obtaining chlorophyll

containing callus tissues from the leaves of lettuce (Lactuca sativa) and leaf petiole of parsley (Petroselinum hortense). In this Laboratory callus tissues have been obtained from the leaves of seedlings of Hollarhena antidysenterica (unpublished).

Flowers:

Callus cultures of flowers have not been reported in continuous culture though different portions of flowers have been cultured and callus obtained. For instance, excised anthers and even pollen itself have been cultured and callus obtained. In 1963 Vasil reported callus formation from the anthers of Cajanus cajan and noticed that callusing was much more common when the anthers were cultured at the tetrad or one celled microspore stage. This callus could be subcultured easily on White's medium supplemented with coconut milk.

Pollen itself has been cultured in vitro and was found to form callus-like masses in the case of Ginkgo biloba and Taxus by Tulecke (1953, 1959) under the influence of 2,4-dichlorophenoxyacetic acid, coconut milk and yeast extract. In 1960 Tulecke reported that plant extracts could be completely replaced by arginine, 1-naphthylacetic acid, calcium pantothenate and a high concentration of phosphate in the medium.

Fruits:

In 1958 Nordan cultured the juice vesicles of lemon and obtained a callus tissue using a synthetic medium containing

mineral salts and sucrose, and in 1963 he reported that more rapid tissue growth could be obtained if ammonia was used in place of nitrate as nitrogen source in the basal nutrient medium. Apple mesocarp has also been cultured successfully by Letham (1958) and Nitsch (1959). Proliferation was found to be dependent upon the simultaneous presence of an auxin such as 2,4-dichlorophenoxyacetic acid, or 2,4-dichlorophenoxyacetic acid together with coconut milk or liquid corn endosperm. These results have recently been confirmed by Letham (1960) with the mesocarp tissues of several pome fruits, especially the pear and quince. He found that asparagine markedly improved growth in conjunction with 2,4-dichlorophenoxyacetic acid. In 1963 Nitsch was also able to confirm the results of Letham (1960) using two different varieties of pears. Callus masses have been obtained from the endosperm tissues of maize by Straus and La Rue (1954) who used filtered yeast extract, which they later (1960) replaced by asparagine in a fully synthetic medium. Mohan Ram and Steward (1964) have also obtained callus growth from a number of different varieties of banana fruit.

In addition to the cultures obtained from different parts of the plant, callus from various plant origins has also been obtained. Morel and Wetmore (1951) obtained callus from the stem portion of Amorphophallus rivieri (a monocot) on a coconut milk-2,4-dichlorophenoxyacetic acid medium and in 1965 Mascarenhas et al have reported callusing in Zea mays

tissues in the presence of diphenylurea in a fully synthetic medium. As a representative of the Gymnosperms may be mentioned the red wood tissue which was cultured by Ball (1950). In the large group of pteridophytes the work of Steeves and Sussex (1952) who obtained in vitro cultures of fern callus both in solid and liquid media containing glucose may be cited. In addition callus cultures have also been obtained from a number of mosses (Ward, 1964).

3) Tumour tissue:

Callus cultures have also been obtained from different types of plant galls and tumours in a bacteria-free condition. White and Braun (1942) suggested that the best material for this work comes from the secondary galls, some of which may be free from the inciting crown gall bacteria. An alternative procedure is to isolate tissue from a rapidly growing crown gall, but in this case only a very small percentage of cultures may be obtained free from microorganisms (Riker and Hildebrandt, 1958). Braun and White in 1943 demonstrated in tissue culture the independent nature of the crown gall tumour cell and that its continuous growth was not dependent upon bacterial stimulation. As a result of the initial bacterial action they found that a genetic change had occurred from a normal to a tumour cell and that the tumour cell could be propagated indefinitely in culture. Moreover when fragments of this tissue were grafted into the original plant they developed typical crown gall without bacteria whereas normal tissues when grafted, fused with

the host and no tumorous growths developed. These observations confirmed the autonomy of crown gall tumours and their independence from bacteria. Gautheret (1955a) made a comparison of normal and crown gall tissue in culture and showed that crown gall tissue, unlike normal tissue, did not require added auxin.

4) Single cell cultures:

The earlier attempts of several workers to grow single cells of plants in vitro were without success, though large inocula could be grown continuously. The observation that callus pieces of some tissues, which were rotated in liquid media, broke up and got dissociated was made by a number of workers, but successful tissue clones from single cells were not obtained until 1954 when Muir et al succeeded in growing single cells by a "nurse culture method". Under aseptic conditions the single cell was picked up under a microscope with a microspatula from liquid or agar cultures and placed on a 8 cm x 8 cm square of sterile filter paper that had rested several days on top of an established nurse tissue growing on agar medium. The nurse tissue piece was either of the same species or from a species different from that of the single cell. If the single cells were transferred directly on the agar medium they failed to grow but with the filter paper nurse method the single cells often divided to produce a mass of cells that grew independently when transferred to the agar medium. Using this method Muir et al (1958) established single cell clones from a number of plant species. Bergmann (1960)

made a considerable advance in the single cell culture technique by utilizing a plating method for individual cells of tobacco and bean. Studies of the separate cells in the agar petri dish were made under the microscope and the cell division was followed by time lapse photography. Jones et al (1960) were able to isolate single cells and follow their growth, maturation, division and even death in a special microculture chamber. Ball (1963) observed single cells during growth and division in 2 mm thick glass plates at the centre of which two half overlapping holes 2.5 cm in diameter were ground.

5) Submerged cultures:

In this technique cultures containing free cells and small clumps of cells are grown in agitated liquid cultures. In 1954 Muir et al obtained such cultures from tobacco callus by agitation in liquid medium on a reciprocating shaker. Similarly in 1956 Reinert obtained suspension culture from Pinus glauca and Nickell in the same year reported that he had continuously subcultured for four years a suspension culture of the hypocotyl of Phaseolus vulgaris. In 1958 Steward et al reported that they had serially subcultured a suspension culture, initially obtained by release of cells from actively growing fragments of carrot root phloem cultured in a specially designed one litre rotating flask containing liquid medium enriched with coconut milk. Some interesting data on the influence of the composition of the medium upon the extent to which separation of cells occurs has also been reported.

Reinert (1956) found that deficiencies of folic acid and vitamin B_{12} increased the friability and dispersion in liquid media of Picea glauca tissues. Similarly Torrey and Shigemura (1957) found that high concentration of yeast extract when combined with a low concentration of 2,4-dichlorophenoxyacetic acid increased dissociation of cells of pea root callus in liquid culture. In 1961 Torrey and Reinert showed that the separation of cells in carrot and Convolvulus arvensis was greatly reduced when 2,4-dichlorophenoxyacetic acid was omitted from the syathetic medium in which they were grown. They also indicated that certain other growth factors, especially choline, ascorbic acid and riboflavin, have a pronounced influence upon cell separation. Very recently Ganapathy et al (1964) have reported on the effect of varying concentrations of boron with ethylenediaminetetraacetic acid, lithium chloride and urea to achieve dissociation of cells.

Though, by modifying the composition of the culture medium a fair degree of dispersion can be obtained they are far from being suspensions of free cells and mostly occur as cell aggregates in which active cell division takes place. Tulecke & Nickell (1959, 1960) with a view to growing large quantities of plant tissues in liquid media, carried out studies in 20-litre carboys containing 9 litres of medium. Under these conditions plant cells can be made to grow like bacteria and these studies can be compared to bacterial fermentations. Lampert (1964) also grew sycamore cells in 5 to 10 litre flasks,

containing 2 to 5 litres of medium. The growth curves he obtained with this tissue could be compared with the typical growth curve obtained with microorganisms. Growing carrot root cells as suspensions in liquid media Mitra et al (1960) could obtain whole carrot plants from single cells present in these suspensions.

Growth factors:

It has now been fairly well established that the failure by the earlier workers to culture plant tissues in vitro was due mainly to the fact that all plant tissues need certain growth factors for their survival and growth, which must be added to the medium. These substances can be classified into different groups.

(a) Auxins: Among the earliest known plant growth regulating substances are the auxins, which play a vital role in the growth of plants. Among some of the more important auxins used in tissue culture, 3-indoleacetic acid, β-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid can be cited. They have been shown to be very active and indispensable for the growth of several plant tissues even when incorporated into the media at very low concentrations.

The different plant tissues which have been grown in culture, can be classified into three main groups depending upon their requirement for auxin and for a new class of plant cell division factors termed "kinins" which include coconut milk

and kinetin (6-furfurylaminopurine).

(1) In the first group those tissues which require both auxin and a kinin can be included. An example of this is the potato tuber which does not proliferate in vitro unless the medium contains both a synthetic auxin like 2,4-dichlorophenoxyacetic acid and a source of cell division factors such as coconut milk (Steward and Caplin, 1951). Skoog and Miller (1957) also observed a similar response with various varieties of tobacco which responded to indoleacetic acid and 6-furfurylaminopurine.

(2) There are some tissues which can grow over prolonged periods in the presence of only an auxin in the mineral salt-sucrose medium and do not require a kinin. The cambial tissues of several species of plants are capable of growing in vitro with an auxin as the sole growth substance (Gautheret, 1959). Growth in this case may be due to the fact that the cambial tissue is capable of producing its own cell division factors and needs only the presence of an added auxin for "cell division". The presence of cell division factors has, however, been confirmed by Nitsch and Nitsch (1956a) in Jerusalem artichoke tissue extracts thereby showing that the absence of a requirement for kinin was due to the ability of the tissue to synthesize it.

(3) There are many tissues which require neither an auxin nor a cell division factor. This is usually the case

with crown gall tissues. The tissue is probably capable of synthesizing both the auxin and cell division factor. The presence of growth promoting factors in crown gall tissue has been shown by Braun and Naf (1954) and also by Braun and Stonier (1958). Gautheret (1939) has also shown that crown gall cultures are able to synthesize their own auxin thus satisfying the dual requirement for an auxin and a cell division factor of the tissues even though the substances are not added to the medium.

Kinetin and kinins:

These substances are another class of growth regulaters and are primarily concerned with the process of cell division. Kinetin (6-furfurylaminopurine) was first isolated from autoclaved herring sperm DNA by Miller et al in 1955. Its effects as a cell division substance in tissue cultures have also been widely tested and reported (Skoog and Miller, 1957; Shantz et al. 1958, Torrey & Shigemura, 1958; Torrey, 1961). Kinetin is not known to occur naturally in plants though "kinins" which designates substances capable of stimulating cell division have been isolated from a number of plants. Lavee (1963) showed the presence of a kinin in autoclaved aqueous extracts of peach fruitlets. Similarly Powell (1964) has reported on the presence of kinins in alcoholic extracts of the endosperm and ovules of Prunus persica and Letham (1963) has shown the presence of kinins in plum fruits and a number of other tissues. A number of other growth promoting substances have

also been reported from many different plants. Khalifah and Lewis (1963) have isolated compounds different from the known indoles or gibberellins from orange fruits. Tomato juice was also shown by Maia (1963) to contain a cell division factor which stimulated growth of explants of Helianthus tuberosus in the presence of auxin and in the same year Zwar and Skoog also obtained promotion of cell division by extracts from pea seedlings. The presence of a kinetin-like compound has also recently been shown in coconut milk Loeffler and Van Overbeek (1963).

Coconut milk, a liquid endosperm, has been used extensively for the culture of a large number of plant tissues (Caplin & Steward, 1948; Henderson et al. 1952; Nickell, 1950) ever since Van Overbeek et al (1941) first used it in their embryo culture studies and found it to have marked effects on growth and cell multiplication. As a result of these findings, attempts were made to isolate the active cell division substances present in coconut milk, especially by Steward and his group. In 1952 Shantz and Steward isolated four unidentified compounds, starting with large amounts of coconut milk and in 1955 they identified one of the compounds as 1-3 diphenylurea which was shown to have a slight growth promoting effect. The separation of active and neutral fractions and of the amino acids of coconut milk has been described by Pollard et al (1961) and Steward et al (1961). Among the components of the neutral fraction sorbitol is present in the greatest quantity and

myo- and scyllo-inositol in smaller amounts. Coconut milk is also rich in phenolic substances, the leucoanthocyanins (Steward et al. 1961). Recently Shantz and Steward (1963) on the basis of studies with coconut milk, extracts of immature grains of Zea mays and Aesculus wurlitzmanis all of which are high in growth promoting activity, have postulated that the growth promoting activity is due to two fractions, a neutral fraction and an active fraction, which act synergistically. Inositol, indoleacetic acid, phenolic bodies such as leucoanthocyanins and nitrogen present in the form of amino acids interact to produce the growth promoting activity. The amino acids can be fully replaced by casein hydrolysate. These studies on the multiple interactions in stimulating cell division and growth were carried out by determining their effect on the growth of explants of secondary phloem tissue of carrot root.

Gibberellins:

Gibberellins and gibberellic acid are a group of growth stimulators which cause marked elongation of internodes of genetic dwarf species of plants. These substances were originally obtained from the fungus "Gibberella fujikuroi" from which it derived its name. The presence of gibberellins or gibberellin-like substances has been shown in many higher plants (Thimann, 1963). Nickell and Tulecke (1959a) tested the effect of gibberellin on a number of different plant tissues in culture and found that they inhibited the growth

of monocotyledonous tissues and promoted growth in a few others while still others showed no response. The gibberellins have profound effects on cell metabolism which include respiration, assimilation and protein and nucleic acid synthesis (Paleg, 1962; Srivastava, 1963; Varner, 1964).

Vitamins:

The earlier studies indicated that vitamins are also necessary for the growth of callus tissue (White, 1943; Gautheret, 1959). Many callus tissues evidently meet most of their vitamin requirements by synthesis since they need only one or a very few vitamins in the medium. In 1939 Gautheret found that vitamin B_1 was favourable for carrot isolates and Nickell in 1952 also found that Rumex callus required this vitamin. Hildebrandt et al (1946a) in studies with sunflower and tobacco tissues found that pyridoxine alone could stimulate the growth of these tissues. Reinert and White in 1956 found vitamin B_{12} beneficial for callus from white spruce. Morel in 1946 found pantothenic acid necessary for Cratagus and Jacquiot (1951) reported a requirement of meso-inositol for elm. De Capite (1952) found that p-aminobenzoic acid stimulated growth of Jerusalem artichoke and Hotson (1953) used ascorbic acid for Juniperis cultures.

Thus we find that plant tissue culture studies can be put to a wide variety of applications. Many plants for instance are known to contain substances of medicinal and

industrial value, such as alkaloids, steroids and antibiotics. Nost and Mika (1957) working with callus cultures of belladonna roots showed the formation of atropine *in vitro*. Nicotine has been isolated from cell cultures of *Nicotiana tabacum* (Speake *et al*, 1964). Reserpine was also found to be present in root callus tissue of *Rauwolfia serpentina* (Mitra and Maul, 1964). French and Gibson (1957) reported that *Datura tatula* root tissue cultures biosynthesize the alkaloids hyoscyamine and hyoscine. Tulecke and Nickell (1960) and Nickell (1962) cultured the avocado cotyledon and noticed that the tissue produced *in vitro* a small amount of antibiotic substance.

Tissue culture studies have also proved of importance in the study of plant morphogenesis. Kinin and other factors have been shown to be important in morphogenesis. Skoog and Miller (1957) with tobacco pith callus tissue were able to study the formation of shoots or roots, by the interaction of kinetin or adenine and an auxin. Similarly Sastri (1963) obtained differentiation in horse radish tissue cultures by variations in the concentrations and by the interaction of kinetin and an auxin. An important advance in the tissue culture field was the demonstration by Steward (1968) that a complete plant could be obtained from dissociated cells of carrot phloem which had been grown *in vitro*.

Large quantities of tissues can also be obtained in liquid media in fermenters (Tulecke and Nickell, 1959, 1960) and these can be treated as a new group of microorganisms with

all the capabilities which they possess. These large quantities of tissues can be used to study biochemical reactions and to produce natural plant products. They may also be used for the study of obligate parasites, fungi and plant nematodes and of plant viruses and tumours (Bergman, 1959). It will be seen from the brief account above that plant tissue and cell cultures are of value in the study of the metabolism of plants, the formation and biosynthesis of alkaloids and other characteristic plant products, the production of antibiotics and in the study of differentiation and plant pathology.

PRESENT WORK

Several plant tissues have been grown *in vitro* in this Laboratory as part of a programme of study of the biochemistry of plant cells. Since the rate of growth of some of these tissues was very low, a few relatively fast growing tissues were examined so that a suitable plant material could be obtained which could be grown rapidly in sufficient quantities for biochemical studies. *Vigna catiang* was selected for detailed study since it showed the maximum rate of growth among all the plant tissues which were examined. Its nutritional requirements and metabolism were investigated and the results of these studies form the subject of this thesis.

Vigna catiang is an annual twining herb, belonging to the family leguminosae, sub-group papilionaceae. It

produces yellow flowers giving rise to seeds which are used for edible purposes. The local name for this plant is 'cholai'.

Callus cultures have been obtained from a number of plants belonging to this family. Nickell in 1956 described the continuous cultivation of callus cultures isolated from the hypocotyl region of the pole bean. This culture was isolated by him in 1951 and maintained on White's medium containing coconut milk and 2,4-dichlorophenoxyacetic acid. It was also grown in submerged culture. Bergmann in 1960 reported on the growth and division of single cells of Phaseolus vulgaris var Early Golden Cluster. The callus was obtained from segments of stem on White's basal medium containing coconut milk and 2,4-dichlorophenoxyacetic acid. In submerged cultures the tissues could also be broken up into single cells each of which could be made to develop into a tissue clone by the plating technique in petri dishes as described earlier. In 1957 Torrey and Shigemura obtained callus from excised root tips of germinated pea seeds, variety Alaska. Beaner's medium with sucrose, yeast extract and 2,4-dichlorophenoxyacetic was used for the growth of this callus tissue. The callus could be grown both in agar medium or in liquid flask culture with constant agitation. Gautheret (1959) has also reported that Nickell (unpublished) obtained callus cultures from stem of Vigna sinensis (Endl) and maintained it on Burkholder and Nickell's (1949) medium containing phosphate, yeast extract and parachlorophenoxyacetic acid.

The main objectives of the present investigation were the following.

1) To obtain viable callus cultures from the hypocotyls of seedlings of *Vigna catiang* and to study the nutritional requirements and establish the optimum conditions for the growth of these cultures on media solidified with agar.
2) To grow the tissue in liquid media in agitated culteres, and to study the rate of utilisation of the nutrients in the medium with a view to obtaining a medium that would give the most rapid growth of tissue.
3) To analyse the tissue for the free and protein amino acids and to compare them with the free and protein amino acids of the intact hypocotyl.
4) To study the effect of different substrates and inhibitors on the respiration of the callus tissue.

Chapter I deals with the materials and the methods used in these studies.

Chapter II describes studies on the nutritional requirements of callus cultures of *Vigna catiang*.

Chapter III deals with studies on the growth of *Vigna catiang* callus tissue in shake flasks and the utilization of some of the nutrients.

Chapter IV describes chemical analyses of *Vigna catiang* callus tissues and a comparison of the free and protein amino acids of the callus tissue and of the intact hypocotyls.

Chapter V deals with studies on the respiration of *Vigna catiang* in the presence of different substrates and inhibitors.

CHAPTER I

MATERIALS AND METHODS

CHAPTER I

MATERIALS AND METHODS

The inorganic salts used for the preparation of media and the substrates for respiration studies were of analytical grade (British Drug Houses or E. Merck). The other chemicals used in the course of the experiments were either gifts or commercial preparations from the sources indicated below in parenthesis:

Yeast extract, malt extract, casein hydrolysate and bacto agar (Difco). Glucose, fructose, sucrose, maltose, arabinose (British Drug Houses). Lactose, galactose, xylose (L.Light & Co.), Glycerol (Merck), sorbitol (Atlas Chemical Co.); glutamine, asparagine (L. Light & Co.), glycine, thiamine hydrochloride (British Drug Houses). Indole-3-acetic acid, methylindol-3yl acetic acid, 2,4,5-trichlorophenoxypropionic acid (British Drug Houses), β-naphthoxy acetic acid, 2,4,5-trichlorophenoxyacetic acid (Nutritional Biochemicals), β-indole-3-propionic acid (Hoffmann La-Roche), 2,4-dichlorophenoxyacetic acid (Scientific & Industrial Supplies Corporation), Kinetin and gibberellic acid (Sigma Chemical Co.). 2-Benzothiozolyloxyacetic acid was a gift from Dr. H. Y. Mohan Ram and the several long chain fatty acids from Dr. S. C. Bhattacharyya. Dowex-50 (200-400 mesh, 8 per cent cross linked) was obtained from the Microchemical Specialities Co., Berkeley and was recycled and converted into the hydrogen form before use. Dowex-1 (200-400 mesh, 8 per cent cross linked) was obtained from Dow Chemical Company and used in the acetate form. Ferric ethylenediaminetetraacetic acid was

prepared from disodium ethylenediaminetetraacetic acid by heating a mixture of aqueous solutions of both in equimolar amounts according to the procedure described by Murashige and Skoog (1962). Coconut milk was obtained by pooling together the filtered water from a number of tender green coconuts and autoclaving at 15 lb for 20 min. It was stored at -20° and thawed and refiltered before use. Vigna catiang seeds were obtained from Pestonjee P. Pocha & Sons, Poona.

A number of different extracts were also made as follows:

1) Rice washings: 20 gm of a local variety of dehusked rice were shaken with 50 ml distilled water for 30 min. The extract was decanted and the washing repeated twice as before with 30 and 20 ml lots of distilled water respectively. The extracts were combined and incorporated in the media at two different levels (10 and 20 per cent by volume).

2) Vigna catiang extract: 45 gm of Vigna catiang tissue grown for 28 days on White's basal-coconut milk-yeast extract-malt extract-2,4-dichlorophenoxyacetic acid medium were ground with acid washed sand in a morter and pestle and the extract filtered through muslin cloth. The residue was reextracted twice with distilled water. The combined extracts were centrifuged and the clear supernatant was made to 100 ml. Supplements of the pooled extract were added at levels of 5 and 10 per cent by volume.

3) Euglena gracilis extract:

Euglena gracilis was grown on a dextrose-liver extract-tryptone medium (0.1 per cent of each) for a week, in a flask under agitation. The alga was then removed by centrifugation and lyophilised. The lyophilised material was extracted successively three times with 20, 15 and 10 ml lots respectively of 60 per cent alcohol and the extracts were pooled. The alcohol was removed by distillation under vacuum. The aqueous residue was extracted three times with diethyl ether to remove colouring matter. The ether extracted aqueous layer (Fraction I) was made up to 10 ml and the pooled ether washings concentrated and (Fraction II) made up to 5 ml. Fractions I and II were incorporated into the growth medium separately as well as in combination.

4) Lemon juice: 250 ml of juice were obtained by squeezing three sweet limes. The juice was filtered through cotton, autoclaved at 15 lb for 20 min, refiltered and incorporated into the medium at a level of 3 per cent by volume.

Composition of media:

The composition of different basal media is described in Tables IA, IB and IC. The nature of the supplements added to the basal media is described in the relevant chapters of the text. White's, Gautheret's and Knop's media were prepared according to the details described by White (1954). Murashige's medium was made according to Murashige and Skoog (1962) and Smith's medium was made up from the formula described in a

TABLE IA

MACRO ELEMENTS

Mineral	White's modified mg/l	White mg/l	Knop mg/l	Murashige mg/l	Gautheret mg/l	Smith mg/l
$Ca(NO_3)_2.4H_2O$	288	288	144	-	100	710
Na_2SO_4	200	200	-	-	-	-
KCl	160	80	-	-	-	-
$NaH_2PO_4.2H_2O$	320	20.4	-	-	-	-
$MnSO_4.H_2O$	4.9	4.9	-	20.0	2.0	-
$ZnSO_4.7H_2O$	24.9	24.9	-	10.0	0.1	-
KNO_3	-	-	25.0	1900	25.0	405
KH_2PO_4	-	-	25.0	170	25.0	70
$MgSO_4.7H_2O$	360	360	51.4	370	25.0	245
NaCl	-	-	-	-	-	60
NH_4NO_3	-	-	-	1650	-	-
$CaCl_2.2H_2O$	-	-	-	440	-	-
$(NH_4)_2SO_4$	790	-	-	-	-	-
$NaNO_3$	180	-	-	-	-	-

TABLE III

MICRO ELEMENTS

Mineral salt	White's modified mg/l	White mg/l	Knop mg/l	Murashige mg/l	Gautheret mg/l	Smith mg/l
H_3BO_3	14	14	-	6.2	1.5	0.57
KI	0.64	0.64	-	0.83	0.5	-
Ferric citrate	5.0	5.0	5.0	-	5.0	-
Ferrous EDTA	5 ml	-	-	5 ml	-	7.2
$MnCl_2$	-	-	-	-	-	0.49
$CaCl_2$	-	-	-	-	-	0.268
Sodium molybdate	0.25	-	-	0.25	-	0.252
$ZnCl_2$	-	-	-	-	-	0.625
$CaSO_4.5H_2O$	0.025	-	0.05	0.025	0.05	-
$Ti_2(SO_4)_3$	-	-	-	-	0.20	-
$NiSO_4$	-	-	-	-	0.05	-
$CoCl_2$	0.025	-	-	-	0.05	-

TABLE 1C

VITAMINS & AMINO ACIDS

	White's modified mg/l	White mg/l	Knop mg/l	Murashige mg/l	Gautheret mg/l	Smith mg/l
Nicotinic acid	5	5	5	0.5	-	0.05
Thiamine HCl	0.1	0.1	0.1	0.1	1.0	0.1
Pyridoxine HCl	0.1	0.1	0.1	0.5	-	0.1
Calcium pantothenate	-	-	-	-	0.1	-
Biotin	-	-	-	-	0.01	-
Inositol	100	-	-	100	-	-
Glycine	3.0	3.0	3.0	2.0	-	3.0
Glutamine	200	-	-	-	-	-
Asparagine	200	-	-	-	-	-
Cysteine HCl	-	-	-	-	10.0	-
Gibberellic acid	1.0	-	-	-	-	-
2,4-Dichlorophenoxyacetic acid	0.6	0.6	0.6	0.6	0.6	0.6
Sucrose	3000	2000	2000	3000	2000	2500
pH	5.8	5.8	5.8	5.8	5.8	5.8

personal communication from Dr. Smith. White's modified medium contained the basic nutrients present in White's medium (1954), in addition to a number of different supplements. In preparing Knop's medium White's 'B' (White, 1954) solution was also included together with ferric citrate and copper sulphate (Tables IA, IB & IC) and Knop's mineral salts.

Glassware:

All glassware used in these studies was of Pyrex brand. Glass articles were routinely cleaned by leaving overnight in sodium carbonate followed by rinsing with tap water and then immersing in 30 per cent nitric acid for 12 hr. The glassware was then washed thoroughly with tap water and later with glass distilled water and dried in a hot air oven at 110°. Test tubes and flasks for growth studies were plugged with cotton, autoclaved at 20 lb for 1 hr and then redried at 110° for 2 hr.

Preparation of media:

For most of the studies White's basal medium was used with the concentrations of salts specified in the tables on the composition of the media. After the addition of the different supplements, the medium was adjusted to a pH of 5.8 to 6.0 using a Beckman model G pH meter and then made to volume. Agar was added at 0.8 per cent concentration and the medium was steamed to dissolve the agar and transferred in 20 ml lots into test tubes (25 mm x 150 mm) or in 30 ml lots into 100 ml conical

flasks. Sterilization was carried out by autoclaving at 15 lb for 20 min followed by steaming for 30 min the subsequent day.

Glutamine, asparagine and inositol were sterilised 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°. The contents of tubes and flasks were then mixed thoroughly and allowed to set. In addition to semi-solid agar medium, liquid medium was also used in some experiments. The medium was added in 75 ml lots to 250 ml Erlenmeyer flasks and sterilisation of the medium was carried out as described earlier.

Inoculation:

All inoculations were carried out in a sterile room under an inoculation hood provided with an ultraviolet lamp. A continuous stream of air which was sterilized by passing through aluminium strips coated with glycerine followed by irradiation with ultraviolet light was passed through the room. Sterile forceps were used for transferring the plant tissues during the inoculations and subcultures. Two pieces of inoculum, each weighing 20 to 30 mg, were transferred to the solid media in test tubes, while three pieces, each weighing 40 to 50 mg, were transferred to 100 ml conical flasks. With liquid media 400 to 500 mg of tissue from actively growing agar cultures were used as the inoculum.

Incubation of cultures:

After inoculation the cultures on agar media were incubated in a room at $25^{0}\pm1^{0}$ and illuminated by diffuse artificial light. The relative humidity of the room was maintained at 60 to 70 per cent. Liquid cultures were transferred to a rotary shaker with 5 mm amplitude and shaken at 100 to 120 revolutions per min.

The period of incubation of all cultures was normally 30 days unless stated otherwise. After incubation the callus pieces were removed carefully from the surface of the agar media and weighed. They were then dried to constant weight at 110^{0} to obtain dry weights. The values recorded in the tables are average weights of 10 explants from 5 test tubes or 15 explants from 5 conical flasks for each set.

Tissue grown in liquid cultures was separated by filtration through Whatman No.1 filter paper. The clear filtrate was used for the analysis of the spent medium, whereas the tissue was gently pressed between folds of filter paper to remove traces of the medium and its fresh weight and dry weight were determined as described before. In liquid cultures two flasks were used for each experiment and the average weight is recorded in the tables.

Stock cultures of the callus tissue were maintained on White's basal medium containing 0.1 per cent yeast extract,

0.1 per cent malt extract, 10 per cent coconut milk, and 0.6 p.p.m. 2,4-dichlorophenoxyacetic acid and agar in test tubes. Subcultures were made every 30 days into fresh tubes containing 20 ml of the above medium. The tissue has not been maintained continuously in liquid media by pipetting the cell suspension to fresh flasks since the tissue forms clumps which are not friable and offer difficulty in subculture.

Estimations:

Nitrogen was estimated in the medium and tissue by the micro-Kjeldahl method, total phosphorus by the procedure of Fiske and Subba Row (1925), calcium by precipitation as the oxalate and titration with permanganate (Hawk & Bergeim, 1944). Iron was estimated colorimetrically according to Sandell (1944) using o-phenanthroline.

The tissue was extracted with hot 70 per cent ethanol according to the procedure of Steward et al (1960) and free amino acids were separated by the method of Plaisted (1958). Protein amino acids were obtained by hydrolysis of the alcohol-insoluble residue with 6N HCl for 20 hr in a sealed evacuated tube followed by removal of the excess acid in a vacuum desiccator over KOH pellets and concentrated sulphuric acid and dissolving the residue in a known volume. The identification and quantitative determination of the amino acids were carried out according to the method of Porter et al (1957) using two dimensional paper chromatography and by comparison with runs carried out using a number

of standard amino acids under identical conditions. The solvent systems used were butanol:acetic acid:water (4:1:5) in the first direction and phenol saturated with water in the second direction (Partridge, 1948).

Free sugars in the extracts were identified and determined by separation on paper chromatograms and comparison with standard sugars. The solvent systems used were butanol: acetic acid:water (4:1:5) and phenol saturated with water (Meinstein et al. 1959).

CHAPTER II

NUTRITIONAL REQUIREMENTS OF V.CATJANG CALLUS TISSUE

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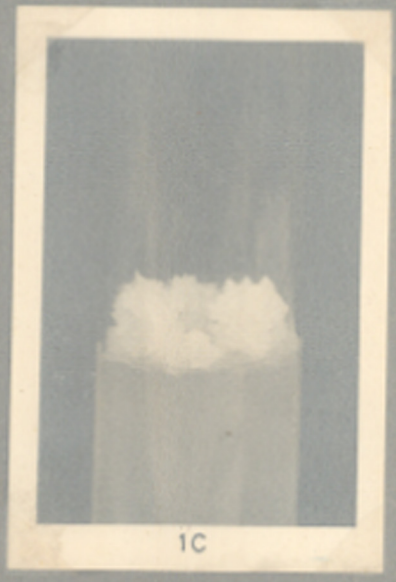
NUTRITIONAL REQUIREMENTS OF *VIGNA CATIANG* CALLUS TISSUE

Intact plants have very few and simple growth requirements, which in most cases can be supplied by the soil in which they have taken root. However, when a tissue from a higher plant is grown on a medium in a glass container, the nutritional requirements are more complex and the tissue shows a requirement for mineral salts, a nitrogen source, a carbohydrate source and often for an auxin and a kinin. This section describes the formation of callus from explants of *Vigna catiang* tissues and its nutritional requirements. When these explants are transferred into different media, their ability to differentiate is lost and the cells start multiplying in a random manner giving rise to masses of undifferentiated cells which are known as "callus" (Plate 1). The optimum conditions for initiation of callus were first determined. After the callus culture was obtained its mineral and carbohydrate requirements, and the action of stimulatory substances, the effect of pH, gas tension and a number of other requirements of the *Vigna catiang* callus tissue were studied. All these growth studies were carried out in media solidified with agar, unless otherwise stated in the respective tables.

1A

1 B



1C

PLATE 1) Callus formation from Vigna unguiculata seedlings

A 6 day old seedling

B Callus forming from a hypocotyl explant (13 days)

C Callus after 30 days' growth.

SECTION I

RESULTS

Initiation of callus:

About 20 *Vigna catiang* seeds were first surface sterilized as follows. The seeds were thoroughly washed with distilled water, immersed in 70 per cent alcohol for 5 min and then washed with distilled water. The seeds were then shaken for five minutes with 50 ml of distilled water containing 8 to 10 drops of a detergent solution (DET). The DET solution 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 min. The hypochlorite solution was discarded and the seeds were washed under aseptic conditions with sterile distilled water till no trace of hypochlorite could be detected. The seeds were then transferred to test tubes or petri dishes containing sterile cotton moistened with sterile distilled water and kept for germination at 25° in the dark. The seeds germinated after 5 to 6 days. When the seeds had germinated, 4 to 5 mm segments from the hypocotyl portions of the seedlings were transferred aseptically into test tubes containing a number of different media combinations (Table 2) and the formation of callus was observed periodically. It is evident that in the early stages, 2,4-dichlorophenoxyacetic acid or naphthalene acetic acid are unnecessary for callus formation, perhaps

TABLE 2

EFFECT OF DIFFERENT SUPPLEMENTS ON THE FORMATION OF CALLUS FROM *VIGNA CATIANG* HYPOCOTYLS

Medium used: White's basal. Supplements added:

CM 15 per cent, NAA 1.0 mg/litre, 2,4-D 0.6 mg/litre,

YE 0.1 per cent, ME 0.1 per cent, agar 0.8 per cent.

Length of inoculum 5 mm (hypocotyl).

20 ml medium/tube.

Supplements added	Result	Period of incubation days	Remarks
1. White's basal	-	40	Hypocotyl dead
2. Coconut milk	+++	40	Does not grow on subculture without 2,4-D
3. CM + 2,4-D	++	40	Grows on subculture
4. CM + YE + NAA or 2,4-D	+++	40	Grows on subculture
5. CM + YE + ME + 2,4-D	+++	40	Grows well on subculture
6. YE + ME + 2,4-D	++	40	Poor growth
7. CM + YE + ME	++++	40	Best medium for isolation of callus

\- no growth
\+ 100-200 mg of tissue/tube
++ 200-400 mg of tissue/tube
+++ 400-700 mg of tissue/tube
++++ 1 gm wet tissue/tube

because of the presence of the auxin in the explants themselves. The medium for optimum callus formation seems to be a combination of coconut milk, yeast extract and malt extract, with the mineral salts, vitamins and sucrose as used by White (1954). It may be noted that though this particular combination is suitable for callus formation it is not suitable for callus growth with repeated subculture since the tissue fails to grow after one or two subcultures unless 2,4-dichlorophenoxyacetic acid is also incorporated in the medium. Coconut milk also appears necessary for the tissue, though a little growth may be observed if yeast and malt extracts are added in its place. The tissue failed to form callus or to grow on White's basal medium without any supplements.

SECTION 2

NUTRITIONAL REQUIREMENTS OF VIGNA CATIANG CALLUS

After obtaining callus tissue from the hypocotyl of *Vigna catiang*, further studies on its nutritional requirements were then carried out. Preliminary results indicated (Table 3) that the callus tissue requires in addition to White's basal medium an auxin, and either coconut milk or kinetin for its continuous growth. The tissue failed to survive on a medium supplemented with either auxin or coconut milk or kinetin alone. The presence of both auxin and coconut milk or auxin and kinetin was essential for this tissue and these components were used in all subsequent studies.

Similarly preliminary studies also indicated (Table 4) that optimum growth of the tissue could be obtained by the addition of yeast extract and malt extract to the White's basal-2,4-dichlorophenoxyacetic acid-coconut milk or kinetin medium. The effect of yeast or malt extract added separately was not as good as when they were added together to the medium. Kinetin was also found to substitute for coconut milk though the growth was less than with coconut milk. Yeast and malt extract were often incorporated in the medium to obtain higher growth rate.

The effect of varying the composition of the basal salts, the carbohydrate and nitrogen source, the kinin and auxin and other supplements was then studied.

TABLE 3

EFFECT OF KINETIN AND COCONUT MILK ON GROWTH OF *VIGNA CATIANG* CALLUS TISSUE

Medium used: White's basal.

Period of incubation: 30 days

Supplement added and concentration	Wet weight mg/tube	Dry weight mg/tube	Remarks
2,4-D (0.6 mg/l)	128	6	Does not survive subculture
C.M. (10 per cent)	334	28	" " " "
Kinetin (1.0 mg/l)	100	5	" " " "
CM (10 per cent)+ 2,4-D (0.6 mg/l)	482	38	Grows on subculture
Kinetin (110 mg/l) + 2,4-D (0.6 mg/l)	200	14	" " " "

TABLE 4

EFFECT OF DIFFERENT SUPPLEMENTS ON THE GROWTH OF *MUSSA CATIANG* TISSUE

Medium: White's basal + 2,4-dichlorophenoxyacetic acid (0.6 mg/l); 30 ml medium used in 100 ml conical flasks.

3 pieces of tissue inoculated per flask.

Period of incubation: 31 days.

	Supplement used and concentration (per cent)	Wet weight mg/flask	Dry weight mg/flask
1	None	210	10
2	Coconut milk (10)	600	30
3	Coconut milk (10) + yeast extract (0.1)	2400	120
4	Coconut milk (10) + malt extract (0.1)	810	40
5	Coconut milk (10) + malt extract (0.1) + yeast extract (0.1)	4200	211
6	Yeast extract (0.1) + malt extract (0.1)	318	16
7	Kinetin (1.0 mg/l) + yeast extract (0.1) + malt extract (0.1)	1040	52

Mineral nutrition:

Several different basal media have been suggested for growing plant cells in vitro and their compositions have been given under Materials and Methods (Tables IA, IB & IC). The growth of Vigna catiang callus was tested on White's, Knop's, Gautheret's, Murashige's and Smith's basal media supplemented with 2,4-dichlorophenoxyacetic acid and either coconut milk or kinetin. The results are described in Fig.1. In the presence of coconut milk all the media except Murashige's gave approximately the same growth. In the presence of kinetin, the growth response was not as good as with coconut milk and was markedly less with Gautheret's or Murashige's or Knops' media than with Smith's or White's media. In all subsequent experiments White's medium was, therefore, used.

The effect of varying the amount of calcium, phosphorus and magnesium in the growth medium and the effect of supplementing with molybdenum, copper and cobalt salts in trace quantities were tested, since the latter are not added to White's medium though they may be present as impurities in the different reagents. This set of experiments was carried out in the presence of coconut milk, yeast extract, malt extract and 2,4-dichlorophenoxyacetic acid (Table 5). Tissue growth on media containing either one-third or one-tenth the calcium nitrate normally present in White's basal medium is only about two-thirds of that obtained with the

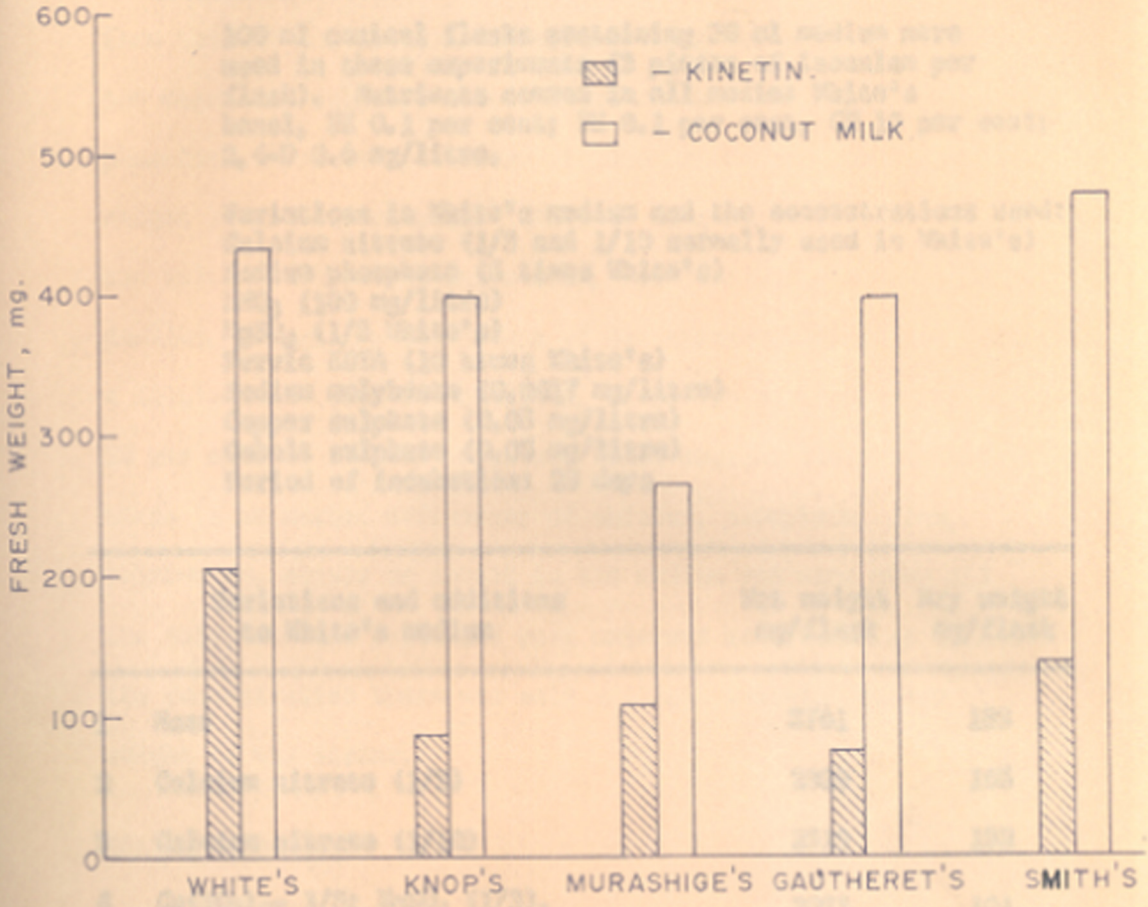


FIG. 1. EFFECT OF DIFFERENT BASAL MEDIA ON GROWTH OF VIGNA CATJANG. PERIOD OF INCUBATION: — 30 DAYS. MEDIUM USED:— DIFFERENT BASAL MEDIA + 2,4-D (0·6 mg/l + COCONUT MILK (10 %) OR KINETIN (1·0 mg/l).

TABLE 5

EFFECT OF VARYING THE CONCENTRATIONS OF SOME OF THE MINERALS AND THE ADDITION OF OTHERS TO WHITE'S BASAL MEDIUM

100 ml conical flasks containing 30 ml medium were used in these experiments (3 pieces of inoculum per flask). Nutrients common in all media: White's basal, YE 0.1 per cent; ME 0.1 per cent, CW 10 per cent; 2,4-D 0.6 mg/litre.

Variations in White's medium and the concentrations used:
Calcium nitrate (1/3 and 1/10 normally used in White's)
Sodium phosphate (5 times White's)
KNO_3 (100 mg/litre)
$MgSO_4$ (1/3 White's)
Ferric EDTA (10 times White's)
Sodium molybdate (0.0017 mg/litre)
Copper sulphate (0.05 mg/litre)
Cobalt sulphate (0.05 mg/litre)
Period of incubation: 28 days

	Variations and additions to White's medium	Wet weight mg/flask	Dry weight mg/flask
1	None	3761	183
2	Calcium nitrate (1/3)	2029	105
3	Calcium nitrate (1/10)	2319	132
4	$Ca(NO_3)_2$- 1/3; $MgSO_4$ (1/3), NaH_2PO_4 (x5), KNO_3, FeEDTA(x10)	2033	101
5	Sodium molybdate, $CuSO_4$ & $CoSO_4$	2775	142
6	As in (4) + sodium molybdate + $CuSO_4$	1790	94
7	MS + YE + ME (double strength)	3210	220

control medium. The growth of the tissue in the presence of trace amounts of molybdenum, copper or cobalt is also less than that of the control. Similarly when the calcium nitrate and magnesium sulphate contents are reduced and excess of phosphate, iron and potassium nitrate are added, the tissue weight is greatly reduced. However when the levels of all the salts in White's basal medium, yeast and malt extracts were doubled, though the wet weight of the tissue obtained was just a little lower than the controls, there was an increase of 20 per cent in the dry weights. These results indicate that there is no marked deficiency of calcium, phosphate, iron, molybdenum, copper or cobalt in the medium and even when all the salts and the yeast and malt extracts were taken at double the concentration there was only a slight increase in dry weight of the tissue.

Nitrogen sources:

In White's medium, the nitrogen requirements are mainly met by calcium nitrate. To find out whether any other nitrogen compound, inorganic or organic, can substitute for nitrate or improve tissue growth, White's basal medium was prepared without calcium nitrate, the calcium content being made up by adding an equimolar amount of calcium chloride. Kinetin was used as a kinin instead of coconut milk since the latter contains several organic nitrogen compounds such as amino acids. All the inorganic nitrogen compounds tested were added

to supply nitrogen at concentrations equivalent to the nitrogen present in the calcium nitrate of White's medium (Table 6). The quantity of nitrogen added as coconut milk, casein hydrolysate and yeast extract was, however, variable and a wide variety of nitrogen compounds was present in these supplements. Ammonium salts gave poor initial growth compared to nitrate and there was no significant difference in growth after 28 days or 49 days indicating that after a slight initial utilization, ammonium salts fail to support further growth. Cyanate supported growth but subsequent experiments showed that the tissue failed to survive repeated subcultures on cyanate as a nitrogen source. Urea and asparagine supported growth though to a lesser extent than nitrate, whereas casein hydrolysate gave very good growth. The total nitrogen added as casein hydrolysate was only 16 mg which is half that present in the control and there was, therefore, no increase in growth after 28 days with casein hydrolysate since the nitrogen was probably limiting. Yeast extract and coconut milk could completely replace the inorganic nitrogen and the tissue growth was better than on the standard medium with calcium nitrate especially after 49 days. Coconut milk was found to contain 0.24 mg of total nitrogen per ml.

Carbon sources

For studying the effect of different carbon sources on the growth of the tissue White's basal-yeast extract-malt extract-2,4-dichlorophenoxyacetic acid medium was used with kinetin instead of coconut milk since the latter contains

TABLE 6

EFFECT OF DIFFERENT NITROGEN SOURCES

Calcium nitrate was omitted from White's basal medium. The calcium deficiency was made up by adding an equivalent amount of calcium chloride. The nitrogen content of the medium in experiments 1 to 8 was the same whereas the nitrogen content in experiments 9, 10 & 11 was variable.

Medium used: White's basal minus calcium nitrate. Kinetin 1.0 mg/litre; 2,4-D, 0.6 mg/litre.

Nitrogen source	Wet weight mg/tube		Dry weight mg/tube	
	28 days	49 days	28 days	49 days
1. Calcium nitrate (control)	275	388	15.0	16.5
2. Ammonium sulphate	201	215	10.5	12.0
3. Potassium cyanate	228	326	12.0	17.5
4. Ammonium chloride	150	184	8.0	12.0
5. Ammonium nitrate	162	179	9.5	11.0
6. Ammonium citrate	69	79	3.5	4.5
7. Asparagine	141	250	7.5	15.0
8. Urea	157	215	8.0	12.5
9. Coconut milk (10 per cent)	216	470	13.5	30.5
10. Casein hydrolysate 200 p.p.m.	249	266	13.0	15.5
11. Yeast extract (0.1 per cent)	339	562	18.5	32.0

several sugars. Different carbohydrates and other carbon sources were incorporated in the media at a 2 per cent concentration and the growth of the tissue was determined (Table 7). Without any carbon source in the medium, the tissue grows very slightly as a brown mass. Very little or no growth was observed with fructose, galactose, xylose, arabinose, ethanol or sorbitol in comparison with the controls. With maltose and glucose there was some stimulation in the growth of the tissue. Glycerol and lactose gave fairly good growth whereas sucrose was superior to all other compounds which were tested.

The optimum sucrose concentration was then determined by growing the tissue on medium containing different levels of sucrose in the presence of kinetin. It will be seen that the tissue growth progressively increases with increasing concentration of sucrose and reaches a maximum at 4 per cent sucrose. The fresh weight of tissue obtained with 6 per cent sucrose is less than that obtained with 4 per cent sucrose, but this may be due only to the higher water content of the latter, since the dry weights of tissue obtained with 4 per cent or 6 per cent sucrose are the same both at 20 days and at 33 days. (Table 8)

The effect of sucrose concentration was then determined with coconut milk as kinin instead of kinetin (Table 9). In contrast to the results obtained in the previous experiments, the optimum concentration of sucrose was 2 per cent and there was a marked decrease in both fresh and dry weight at 20 days or 33 days with 1 per cent or 3 per cent sucrose. Glucose, sucrose

TABLE 7

EFFECT OF DIFFERENT CARBON SOURCES

Medium used: White's basal (except for the carbohydrate source); YE (0.1 per cent); ME (0.1 per cent), kinetin (1.0 mg/litre); 2,4-D (0.6 mg/litre). Carbon sources tested were added at a 2 per cent concentration.

Carbon source used	Wet weight mg/tube		Dry weight mg/tube	
	27 days	34 days	27 days	34 days
1. None	84	109	3	3.5
2. Sucrose	269	434	17	26
3. Glucose	75	166	4.5	10
4. Fructose	40	93	3.5	5.5
5. Lactose	193	220	10.5	16.5
6. Maltose	103	142	6.0	8.5
7. Galactose	35	39	3.0	3.5
8. l-Arabinose	35	71	2.5	4.0
9. l-Xylose	12	22	1.0	1.5
10. Sorbitol	81	94	5.0	5.5
11. Glycerol	226	232	12.5	13.0
12. Ethanol	28	54	1.5	2.5

TABLE A

EFFECT OF DIFFERENT SUCROSE CONCENTRATIONS ON TISSUE GROWTH WITH KINETIN

Medium used: White's basal (with ranging sucrose concentrations); YE (0.1 per cent); ME (0.1 per cent); Kinetin (1.0 mg/litre); 2,4-D (0.6 mg/litre).

Concentration of sucrose (per cent)	Wet weight mg/tube		Dry weight mg/tube	
	20 days	33 days	20 days	33 days
1. No sucrose	106	148	4.0	5.0
2. 0.1	167	186	5.0	6.0
3. 0.5	139	240	4.5	10.0
4. 1.0	170	401	7.5	18.0
5. 2.0	197	467	10.0	26.0
6. 2.5	207	575	12.5	37.5
7. 3.0	216	695	15.0	48.5
8. 4.0	575	952	44.0	71.0
9. 6.0	427	708	45.0	72.0

TABLE 9

EFFECT OF DIFFERENT SUCROSE CONCENTRATIONS ON TISSUE GROWTH WITH COCONUT MILK

Medium used: White's basal + CM (10 per cent); YE (0.1 per cent); ME (0.1 per cent); 2,4-D (0.6 mg/l).

Concentration of sucrose	Wet weight mg/tube		Dry weight mg/tube	
	20 days	33 days	20 days	33 days
1 per cent	517	1521	30	78
2 per cent	1315	2230	80	122
3 per cent	561	1435	32	72

sorbitol and inositol are present in coconut milk and it is not known whether they have any effect on the lower optimum concentration of sucrose which was observed in coconut milk media.

Coconut milk

In all the experiments coconut milk has been added at a 10 per cent concentration. Fig. 2 shows the results of varying the concentrations of coconut milk in the medium containing White's basal salts, yeast and malt extracts and 2,4-dichlorophenoxyacetic acid. On this medium, there was some growth,

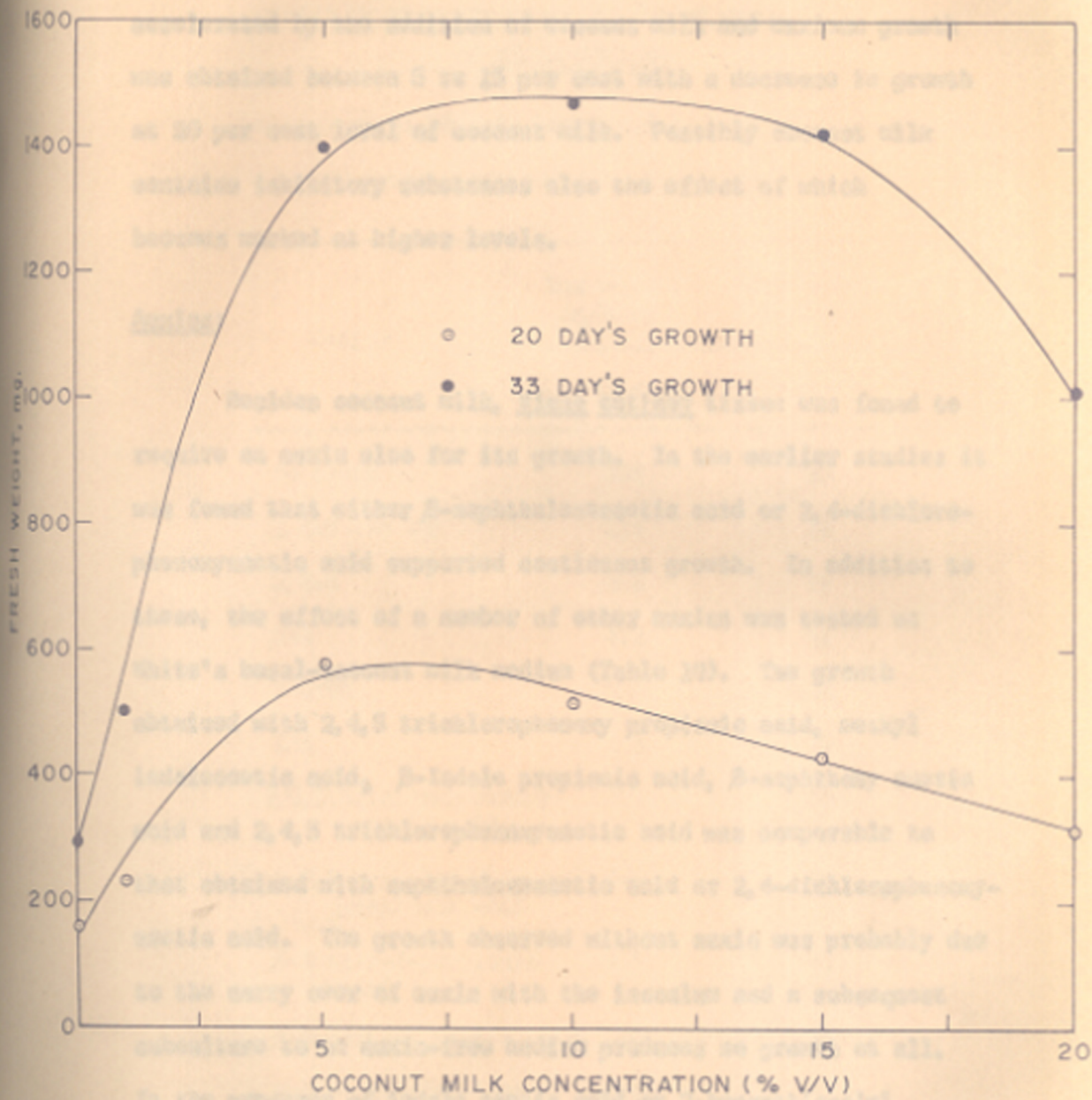


FIG. 2. EFFECT OF DIFFERENT CONCENTRATIONS OF COCONUT MILK. MEDIUM USED:— WHITE'S BASAL, YEAST EXTRACT (0·1 %), MALT EXTRACT (0·1 %) 2,4-D (0·6 mg/l)

even in the absence of coconut milk, but growth was markedly accelerated by the addition of coconut milk and maximum growth was obtained between 5 to 15 per cent with a decrease in growth at 20 per cent level of coconut milk. Possibly coconut milk contains inhibitory substances also the effect of which becomes marked at higher levels.

Auxins:

Besides coconut milk, Vigna catiang tissue was found to require an auxin also for its growth. In the earlier studies it was found that either β-naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid supported continuous growth. In addition to these, the effect of a number of other auxins was tested on White's basal-coconut milk medium (Table 10). The growth obtained with 2,4,5 trichlorophenoxy propionic acid, methyl indoleacetic acid, β-Indole propionic acid, β-naphthoxy acetic acid and 2,4,5 trichlorophenoxyacetic acid was comparable to that obtained with naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. The growth observed without auxin was probably due to the carry over of auxin with the inoculum and a subsequent subculture to an auxin-free medium produces no growth at all. In the presence of indole acetic acid or 2-benzothiozolyl oxyacetic acid the growth was poor and similar to or inferior to that on White's basal-coconut milk medium without auxin. The tissue was also brown and subcultures could not be maintained on media containing these auxins.

TABLE 10

EFFECT BETWEEN DIFFERENT AUXINS ON GROWTH OF VIGNA CATJANG

Medium used: White's basal and Coconut milk 10 per cent.

All the auxins were added at 1 mg/litre.

Medium	Wet weight mg/tube	Dry weight mg/tube
	30 days	30 days
No auxin	334	29
2,4-D (No CM)	113	12
2,4-D	482	49
NAA	472	31
2-BTOA	320	24
IAA	203	16
2,4,5-trichlorophenoxy-acetic acid	435	30
2,4,5-trichlorophenoxy-propionic acid	546	30
β-Indole-3-propionic acid	450	31
β-Naphthoxy acetic acid	431	34
Methyl indole acetic acid	527	33

Fatty acids:

Several fatty acids such as oleic acid have been shown to have a growth-promoting effect on bacteria (Hofmann et al. 1957) and a dibasic aliphatic acid (traumatic acid) has been reported to enhance growth of bean pod tissue (Davies, 1949a). The effect of several long chain fatty acids on the growth of Vigna catiang callus was then determined in two separate sets of experiments. In the first these compounds were added to White's basal-yeast extract-malt extract-2,4-dichlorophenoxy-acetic acid medium to determine whether they could replace coconut milk as a kinin for this tissue (Table 11). These fatty acids were tested at two concentrations, but the growth was markedly inferior even to the growth obtained in the control media without coconut milk. These compounds cannot, therefore, replace coconut milk or kinetin for this tissue. In the second set of experiments these compounds were added at different concentrations to White's basal-yeast extract-malt extract-2,4-dichlorophenoxyacetic acid medium containing coconut milk in order to determine whether they could enhance growth even though they cannot replace coconut milk. It is evident from Table 12 that none of the unsaturated fatty acids tried could improve the overall growth of Vigna catiang tissue above that of the controls. On the contrary with oleic and linoleic acids the growth is definitely inhibited and is lower than with the control medium.

TABLE 11

EFFECT OF FATTY ACIDS ON GROWTH OF *VIGNA CATIANG* IN THE ABSENCE OF COCONUT MILK

Medium used: White's basal; YE (0.1 per cent); ME (0.1 per cent); 2,4-D (0.6 mg/litre).

Fatty acid (concentration)	Wet weight mg/tube	Dry weight mg/tube	Period of incubation days
1. Medium control	210	11	
2. Undecylenic acid (1 mg/l)	127	7	30
3. Undecylenic acid (5 mg/l)	57	3	30
4. ω -Hydroxy palmitic acid (1 mg/l)	64	4	30
5. ω -Hydroxy palmitic acid (5 mg/l)	105	6	30
6. ω -Hydroxy stearic acid (1 mg/l)	105	7	30
7. ω -Hydroxy stearic acid (5 mg/l)	59	4	30
8. ω -Hydroxyhexadecenoic acid (1 mg/l)	75	4	30
9. ω -Hydroxyhexadecenoic acid (5 mg/l)	36	2	30
10. ω -Hydroxy pentadecanoic acid (1 mg/l)	58	2.5	30
11. ω -Hydroxy pentadecanoic acid (5 mg/l)	91	4.0	30

TABLE 12

EFFECT OF FATTY ACIDS ON GROWTH OF *VIGNA CATIANG* ON A MEDIUM CONTAINING COCONUT MILK

Medium used: White's basal; YE (0.1 per cent); ME (0.1 per cent); CM (10 per cent); 2,4-D (0.6 mg/litre)

Fatty acid mg/litre	Wet weight mg/tube		Dry weight mg/tube	
	23 days	33 days	23 days	33 days
1. Medium control	1308	2179	79	94
2. ω-Hydroxypalmitic acid (1.0)	1544	2139	100	96
3. ω-Hydroxypalmitic acid (5.0)	1310	2083	88	118
4. ω-Hydroxystearic acid (1.0)	1343	2182	88	122
5. ω-Hydroxystearic acid (5.0)	1091	1887	70	96
6. ω-Hydroxy hexadecanoic acid (1.0)	1211	1843	71	102
7. ω-Hydroxy hexadecanoic acid (5.0)	949	2000	61	83
8. ω-Hydroxy penta decanoic acid (1.0)	1265	1789	80	82
9. ω-Hydroxy penta decanoic acid (5.0)	1384	1876	88	85
10. Oleic acid (1.0)	544	854	35	54
11. Linoleic acid (1.0)	620	1022	40	62

Plant extracts:

Aqueous and alcoholic extracts of a number of plant materials have been found to contain kinins and other growth substances (Maia, 1963; Letham, 1963). The effect of a number of plant extracts as substitutes for coconut milk was, therefore, tested on Vigna catjang callus cultures (Table 13). The methods of extraction are given under Materials and Methods. None of the extracts tested showed any stimulation when compared with the controls and growth was markedly less than that obtained with coconut milk. Vigna catjang callus extracts were inhibitory at both the levels of 5 and 10 per cent (v/v) which were used in the tests.

Effect of pH:

Figure (3) shows the effect of pH on the growth of the tissue in White's basal-coconut milk-yeast extract-malt extract-2,4-dichlorophenoxyacetic acid medium. The initial pH of the medium was adjusted to the required value by the addition of 0.1N sodium hydroxide or hydrochloric acid. It will be seen that there is an increase in the growth of the tissue as the initial pH of the medium is increased from 4.0 to 6.0. There is however a decrease in growth as the pH is increased from 6.0 to 9.0. At pH 9.0 the tissue does not survive.

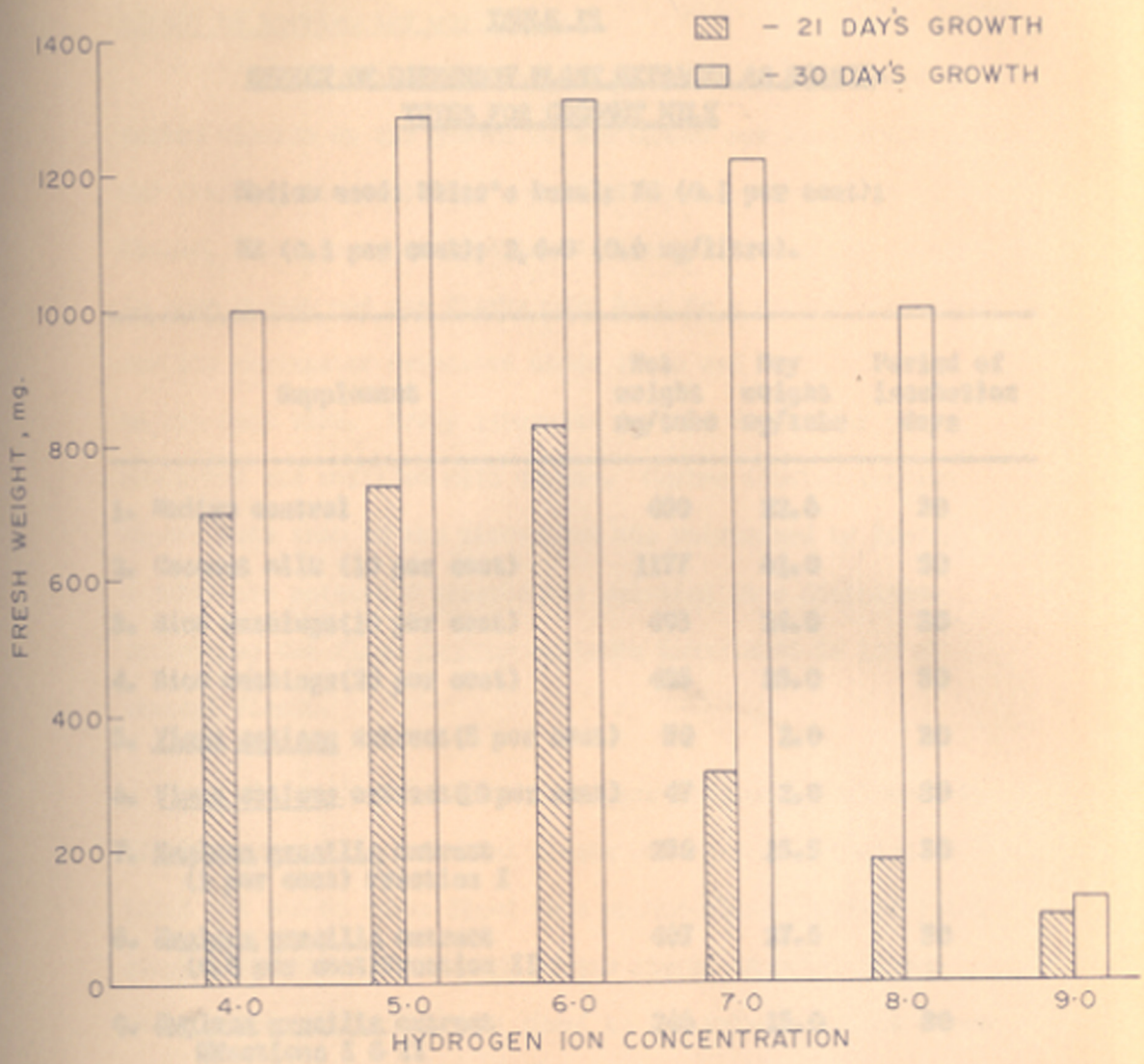


FIG. 3. EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH OF VIGNA CATJANG. MEDIUM USED:— WHITE'S BASAL + COCONUT MILK (10 % V/V). YEAST EXTRACT (0·1 %) + MALT EXTRACT (0·1 %) + 2,4-D (0·6 mg/l)

TABLE 13

EFFECT OF DIFFERENT PLANT EXTRACTS AS SUBSTITUTES FOR COCONUT MILK

Medium used: White's basal; YE (0.1 per cent); ME (0.1 per cent); 2,4-D (0.6 mg/litre).

Supplement	Wet weight mg/tube	Dry weight mg/tube	Period of incubation days
1. Medium control	480	22.6	30
2. Coconut milk (10 per cent)	1177	61.0	30
3. Rice washings(10 per cent)	493	24.0	30
4. Rice washings(20 per cent)	485	23.0	30
5. *Vigna catiang* extract(5 per cent)	50	2.0	30
6. *Vigna catiang* extract(10 per cent)	47	2.0	30
7. *Euglena gracilis* extract (1 per cent) Fraction I	250	15.5	30
8. *Euglena gracilis* extract (0.5 per cent)Fraction II	467	27.5	30
9. *Euglena gracilis* extract Fractions I & II (0.5 per cent each)	246	15.0	30
10. Lemon juice extract (3 per cent)	475	24.0	30

Effect of varying the gas phase:

The effect of the partial pressure of oxygen and of carbon dioxide on the growth of the tissue was then determined. For studying the effect of oxygen tension on the growth of the tissue, the tissue was inoculated into 100 ml conical flasks on agar medium and the flasks were kept in a vacuum desiccator and the desiccator evacuated three times and refilled with oxygen each time. Every alternate day the desiccator was evacuated and refilled with oxygen. Carbon dioxide tension higher than that in the atmosphere was maintained by leaving a saturated potassium bicarbonate solution in a desiccator containing the Vigna cultures inoculated in 100 ml conical flasks.

To study the effect of oxygen tension in cultures in liquid media, 250 ml conical flasks were prepared having an inlet and outlet tubes fixed to a ground-glass standard joint stopper attached to the neck of the flask. A metal filter packed with cotton wool was fixed to the inlet tube and oxygen from a cylinder was passed through this filter. The outlet tube was also provided with a cotton filter plug. The whole apparatus was sterilised before the experiment. The inlet tube extended to about 3/4th the way down the flask and reached almost to the level of the medium and oxygen was passed every day into the flasks and the flasks were left on the rotary shaker. In the shake flasks inoculum weighing 250 to 300 mg

fresh weight was used whereas in the semi-solid medium, three pieces of inoculum having a combined weight of 90 to 100 mg was used.

From Table 14 no significant difference can be seen in the tissue grown on semi-solid media under a high oxygen or carbon dioxide tension and the tissue grown in the laboratory atmosphere. However in the liquid media tissue growth in flasks receiving oxygen was nearly double that obtained in the air controls.

TABLE 14

EFFECT OF VARYING THE GAS PHASE DURING GROWTH OF VIGNA CATJANG

Medium used: White's basal; YE (0.1 per cent); ME (0.1 per cent); CW (10 per cent); 2,4-D (0.6 mg/litre). Period of incubation 40 days. (30 ml agar medium and 75 ml liquid medium added to flasks).

		Wet weight mg/flask	Dry weight mg/flask
	AGAR MEDIUM		
1	Control (air)	3732	198
2	Oxygen	4230	192
3	Control (air)	3400	194
4	Carbon dioxide	3500	195
	LIQUID MEDIUM		
5	Control (air)	7670	375
6	Oxygen	13153	618

Rate of growth:

Growth curve studies using White's basal with coconut milk, yeast extract, malt extract, 2,4-dichlorophenoxyacetic acid media (Fig.4) reveal that *Vigna catiang* tissues follow the usual growth pattern with a lag phase when only a slight growth increase is observed till about the tenth day followed by rapid increase in growth till about the twentyfifth day. After this period there is a levelling off in the growth curve.

Choice of inoculum:

As the callus tissue begins to grow on the medium, visible differences are observed in the color of the tissue in contact with the medium and the portions growing upwards into the tube away from the medium. The upper portions of the tissue show distinct whitish outgrowths when compared with the pale yellow tissue in contact with the medium. Inoculum chosen from the upper and lower portions of the tissue and also at random were transferred to fresh media to determine whether different portions of the tissue would have similar growth rates on subculture. In one such experiment a portion of the medium was also scooped out together with some of the tissue in contact with it and transferred to fresh medium to see if a conditioned medium would help the tissue to grow without the lag phase. In this experiment the inoculum was used from an actively growing 19 day tissue (Table 15). However no significant differences could be observed in the rates of growth of

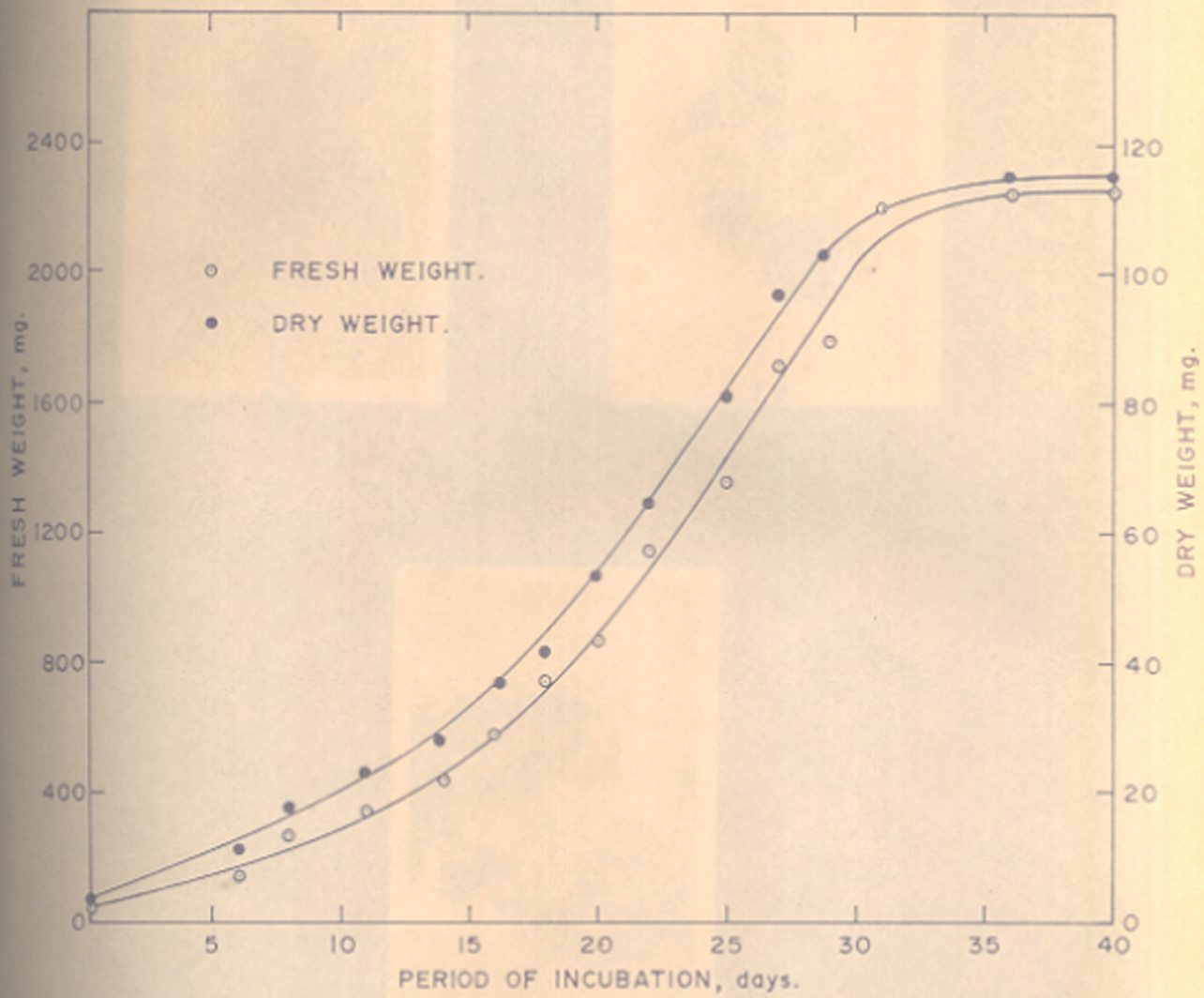


FIG. 4. GROWTH CURVE OF VIGNA CATJANG ON WHITE'S BASAL + COCONUT MILK (10% V/V) + YEAST EXTRACT (0·1%) + MALT EXTRACT (0·1%) + 2, 4-D (0·6 mg/l) (AGAR MEDIUM)

PLATE 2: Microphotographs of acute carcino squashes of 5 day old *Lupin cotinus* callus tissues.

A Cells in metaphase

B Coenocytic cells (showing 2 nuclei per cell)

C Intact cell with non dividing nucleus

the different samples of inoculum (1, 2 and 3 of Table 15). There was a 30 per cent increase in the weight of tissue after 21 days when the medium was also transferred along with the lower portion of tissue as inoculum. This suggests that the medium on which the tissue is grown contains some growth stimulating substances. There was no difference in growth after 36 days possibly due to one or more of the nutrients being exhausted.

Microscopic examination:

Callus cultures of Vigna catiang tissue grown on White's basal-yeast extract-malt extract-coconut milk and 2, 4-dichlorophenoxyacetic acid media were found to be very friable and could be easily broken with very slight pressure or with gentle teasing to give a number of single cells.

Studies on 18 day old tissue showed predominantly very large vacuolated, differentiated, non-dividing cells of various shapes and sizes (average size varying from 100 to 200 mu in length and from 50 to 100 mu in breadth). Various patterns of wall thickenings could be observed at this stage. Since no cell divisions were noticed, a 5 day old culture was used to determine the nature of cell division. Aceto carmine squashes were prepared and used for these observations. It was found that there was considerable variation

TABLE 15

EFFECT OF CHOICE OF INOCULUM

Medium used: White's basal; CH (10 per cent);
YE (0.1 per cent); ME (0.1 per cent);
2,4-D (0.6 mg/litre).

Portion of tissue used	Wet weight mg/tube		Dry weight mg/tube	
	21 days	36 days	21 days	36 days
1. Upper portion of tissue away from the medium	905	2557	51	132
2. Lower portion of tissue in contact with medium	1042	2097	63	143
3. Randomly selected tissue	1100	2779	64	140
4. Lower portion of tissue scooped with some media	1315	2230	76	114

in cell size though a majority of them showed fairly dense cytoplasm and large nuclei. Clear metaphase stages could be observed in some of the cells indicating the occurrence of active cell division at this stage (Plate, 2A). Another interesting aspect which needs further careful observations is the occurrence of coenocytic cells (Plate, 2B). In Plate 2C an intact cell with non-dividing single nucleus can be seen.

SECTION 3

DISCUSSION

Callus cultures were readily obtained from *Vigna catiang* hypocotyls. White's basal medium containing coconut milk was suitable for obtaining callus tissue. It was observed that callus formation was induced on media containing no auxin (Table 2), though for the continuous growth of the tissue auxin was found to be necessary. The initial growth response in the absence of added auxins is probably due to the carry over of the traces of auxin which were present in the hypocotyls themselves.

The continuous maintenance of this callus tissue was found to require two other growth factors in addition to White's basal medium. 2,4-Dichlorophenoxyacetic acid and a kinin, either coconut milk or kinetin being essential for *Vigna catiang* callus. (Table 3). Several plant tissues cannot grow on a medium containing auxin alone but require in addition coconut milk, the two growth factors acting synergistically. Steward and Caplin (1951) have reported that potato tissues showed a marked synergism for coconut milk and 2,4-dichlorophenoxyacetic acid. Mitra and Steward (1961) found a similar effect with their *Haplopappus* cultures. In this Laboratory callus cultures obtained from opium tissues (Sayagaver, 1965) were found to show a synergism for coconut milk and 2,4-dichlorophenoxyacetic acid. *Vigna catiang* callus cultures resembles these tissues in its requirement for both auxin and kinin for continuous growth.

White (1934) obtained unlimited growth of tomato root tips on media containing yeast extract. Since then yeast extract has been used for callus cultures of fern (Steeves and Sussex, 1952), for pollen cultures of Ginkgo biloba (Tulecke, 1953) and for a number of other tissues. Malt extract has been used by Loewenberg and Skoog (1952) for the vigorous and continuous growth of Pinus strobus and by Steinhart et al (1961) for spruce tissues. In the studies with Vigna catiang callus tissue (Table 4) the addition of both yeast and malt extracts were found to result in a marked stimulation of growth especially in a coconut milk medium. The nature of the factors present in these supplements which stimulate growth is, however, not known.

The mineral composition of the medium plays a very important role in the growth of different plant tissues and organs and in 1949 Heller verified that plant tissues are very sensitive to mineral deficiencies. The medium utilised by White (1939a) for his root culture investigations has been found to be satisfactory for a number of plant tissues. In this Laboratory opium tissue was found to grow well on White's basal medium and Mitra and Steward (1961) also obtained callus growth from Haplopappus on this medium. Smith (personal communication) using slightly modified medium was able to grow apple, mermaid rose and a number of other tissues. Murashige and Skoog (1962) obtained a marked stimulation of growth of their tobacco cultures on a mineral salt medium having a higher salt concentration. Gautheret's medium with Berthelot's trace elements has also been used successfully from the time that

Gautheret obtained his initial cultures of carrot in 1939. Nobecourt (1939) in his early studies has also used this medium. Hildebrandt, Riker and Duggar (1946a) adopted the "triangle method" for the determination of the optimum concentration of salts for their sunflower and tobacco crown gall tissues. The present investigations on Vigna catiang tissues indicate that optimum growth is obtained either with White's or with Smith's mineral solution (Fig.1).

Riker and Gutsche (1948) first attempted to study the replacement of certain ions in the medium and established that nitrate cannot be replaced by nitrite. Similarly Burkholder and Nickell (1949) working with tumour tissues reported the superiority of nitrates over the ammonium salts and this was later confirmed by Heller for normal tissues. A superiority of nitrates to ammonium salts was also found in the present studies with Vigna catiang cultures (Table 6). In the first subculture growth was also observed with cyanate as a nitrogen source but the tissue failed to survive on this nitrogen source in subsequent transfers to fresh cyanate containing medium.

The effective replacement of nitrate by organic nitrogen compounds has been reported in several instances in the literature. Nickell and Burkholder (1950) and Frank et al (1951) found that urea allows excellent growth, sometimes superior even to that with nitrate. Riker and Gutsche (1948) have also tried the effect of replacing nitrate by a number of

amino acids and complex substances like peptone, casein hydrolysate and yeast extract and found that although most of the amino acids cannot be utilised some of the amino acids, such as alanine, glycine, arginine, aspartic acid, glutamic acid and asparagine can replace nitrate to a certain extent, whereas complex mixtures like yeast extract and casein hydrolysate are well utilised by sunflower crown gall tissues. In our studies with *Vigna catiang* callus tissue, yeast extract or casein hydrolysate or coconut milk as the sole nitrogen source gave excellent growth which was superior to that with nitrate. The growth with casein hydrolysate was high after 28 days but was not significantly higher at 49 days possibly because the amount of casein hydrolysate was limiting. Asparagine and urea supported growth to a lesser extent than was obtained with nitrate (Table 6).

The early investigators in the field of *in vitro* plant cultures believed that by culturing chlorophyllous organs they could grow tissues without any carbon source. However, they were unsuccessful and all plant tissue cultures have been grown only in media containing a suitable carbon source, the two most commonly used sugars being glucose or sucrose. In 1941 Gautheret compared the nutritive value of sucrose with that of several other sugars at equimolar levels for the growth of carrot tissue. He found sucrose to be the best carbohydrate source followed by glucose, maltose and raffinose. Fructose and galactose were less effective and mannose and lactose were the least efficacious.

In 1953 Ball found that fructose was the best carbon source for Sequoia tissues and that sucrose partially inverted by autoclaving allowed better growth than sucrose. Loewenberg and Skoog (1952) and de Torok and Thimann (1961) with pine tissues used 2 per cent sucrose. Ball (1955) found 3 per cent sucrose to be the optimum for Sequoia cultures whereas Risser and White (1964) found 5 per cent sucrose to be the optimum concentration for spruce tissue cultures. Sucrose was the best carbohydrate source for Vigna antiang callus cultures. Lactose, glucose and maltose also permitted growth, though at a slower rate than with sucrose. Galactose, arabinose and xylose gave very little or no growth (Table 7). The optimum concentration of sucrose in a kinetin medium was 4 to 6 per cent (Table 8) but in a coconut milk medium the optimum sucrose concentration was two per cent and the growth was markedly less at one or three per cent sucrose (Table 9). Further work will be needed to determine whether the marked difference in the optimum sucrose concentration with the two kinins is due to a difference in their mode of action or due to other factors present in coconut milk.

Nickell and Burkholder (1950) tested the effect of sorbitol, inositol and mannitol as sole carbohydrate sources and found that these compounds could not maintain growth on any of the tissues tested. In 1948 Gautheret found that glycerol as the sole carbon source gave good growth when normal carrot tissue was grown in the dark. However, he found that this tissue could not survive subculture. Similarly Hildebrandt

and Riker (1949) studying the effect of alcohols as carbon sources found that methanol, ethanol and propanol could not sustain growth of tumour tissues. Only glycerol sustained growth appreciably but was clearly inferior to glucose and sucrose as a nutrient. In the present work with Vigna catiang cultures also, glycerol is the only polyhydric alcohol having an appreciable nutritive value. Sorbitol and ethanol were not able to support growth of this tissue (Table 7)

The use of coconut milk was first introduced successfully by Van Overbeek et al (1941) for in vitro cultures of embryos of Datura. In 1943 Caplin and Steward cultured carrot phloem fragments in media containing coconut milk and found that autoclaving of the liquid had no deleterious effect on its potency. The most effective levels were found to be from 5 to 20 per cent by volume of the media (Muhamet, 1959). Vigna catiang callus cultures also show a requirement for coconut milk, the optimum concentration being 5 to 15 per cent (volume by volume) (Fig.2).

Auxins are now generally used in the cultivation of normal tissues. Morel and Wetmore (1951) used naphthalene acetic acid for their Anacamptophallus cultures. Jagendorf and Nayler (1952) found that para-chlorophenoxyacetic acid causes callus formation in cabbage roots and Reinert and White (1956) employed 0.1 mg per litre of 2,4-dichlorophenoxyacetic acid for their cultures of spruce cells. In 1964 Risser and White found that for the growth of spruce callus, β-naphthoxyacetic acid was

capable of replacing 2,4-dichlorophenoxyacetic acid in the nutrient medium. Thus different tissues show different auxin requirements. In studies with Vigna catiang the optimum growth was observed with several auxins, 2,4,5 trichlorophenoxyacetic acid, 2,4,5 trichlorophenoxy propionic acid, methyl indole acetic acid, β-naphthoxyacetic acid, α-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. Indole acetic acid and 2-benzothiozolyloxyacetic acid were, however, inhibitory (Table 10).

In 1935 Gautheret tried to replace the sugars in the growth medium of carrot tissues with oxalic, pyruvic and tartaric acids and found them to be toxic even at 0.2 per cent concentrations. Similarly Hildebrandt and Riker (1949 and 1953) tried the effect of sixteen different organic acids on their tissue cultures and established that in general their nutritive value was insignificant. They also tried adding the organic acids at 0.5 per cent concentration in conjunction with sucrose, but obtained no added growth with their tissue cultures. A number of long chain fatty acids was added to the media in conjunction with sucrose and their effect tried on Vigna catiang cultures in our studies. None of the fatty acids tested could replace coconut milk in the medium and the growth obtained in their presence was even less than that obtained in the controls without coconut milk (Table 11). Moreover none of the acids tested with coconut milk stimulated growth more than in the controls and linoleic and oleic acids were found to be slightly inhibitory to growth (Table 12).

Certain extracts from seeds, fruits or stem are known to possess cell division properties. In 1951 Nitsch noticed that tomato juice, like coconut milk, stimulates crown gall tissue proliferation. Maia (1963) isolated from tomato juice a cell division factor capable of stimulating growth of explants of Helianthus tuberosus in the presence of auxin. Similarly autoclaved aqueous extracts from peach fruitlets were found by Lavee (1963) to contain natural kinins. Miggans (1954) found that the juice of ground carrot leaves stimulates the proliferation of this species even after indole acetic acid and other organic acids of the juice are previously eliminated. On the contrary he found root extracts to be toxic to carrot tissues. In our studies on Vigna catiang tissue it was observed that aqueous or alcoholic extracts of rice, algae and lemon were ineffective in promoting growth of the callus and the growth was only equivalent to that obtained in controls with no coconut milk supplement and very much lower than that of tissues receiving coconut milk. An extract prepared from the callus cultures of Vigna catiang itself not only had no kinin activity but was in fact inhibitory (Table 13).

Hildebrandt et al (1955) 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 4.5 and 6.0. White and Risser (1964) have found that spruce tissues have a pH optimum between 5.5 to 6.5. Vigna catiang cells in our studies have been shown to have a broad pH optimum between

5.0 to 7.0 (Fig.3).

A free access of atmospheric air has been shown to be important for the growth of tissue cultures. Thus surface cultures on an agar medium are used in preference to stationary submerged cultures in order to ensure adequate aeration. White in 1953 had shown that if carrot cultures are removed, weighed and returned to the same substrate at regular intervals, greater growth rates could be obtained from those left undisturbed. But in 1964 White and Risser with similar experiments on spruce tissues found no marked effect and concluded that aeration is not necessarily a limiting factor in this case. When Vigna catiang cells were grown on agar either in pure oxygen or in air containing a high carbon dioxide content (Table 14) no significant differences could be observed in the growth of these cultures and those grown in air. Neither oxygen nor carbon dioxide is limiting in agar cultures. In liquid media however, oxygen was found to stimulate growth to a marked degree. The amount of inoculum used in these studies was greater than in agar cultures and the rate of diffusion of oxygen may be limiting so that better growth was observed in oxygen than in air. Several plant cultures have been shown to have a growth curve similar to that of microorganisms with an initial lag phase, a period of rapid growth, a subsequent period of slow growth and then a levelling off in growth. Spruce tissues of White and Risser (1964) showed a lag period for about 5 days followed by the period of maximum growth between the

fifth and the twentyeighth day. Cultures of Vigna catiang callus grown on agar media (Fig.4) show an initial lag period of about ten days, a period of maximum growth till about the end of the fourth week and then a levelling off of growth.

White and Risser (1964) in exhaustive studies with spruce tissues have found that scrambling of cultures before inoculations into fresh media, could greatly reduce the variable results obtained with tissue cultures. When Vigna catiang inoculum was chosen from different portions of the stock callus and subcultured into fresh media (Table 15), no difference in the overall growth rates could be observed but when the medium was also transferred along with the lower portion of the tissue as inoculum, a 30 per cent increase in the weight of the tissue was noticed after 21 days suggesting perhaps the presence of some stimulatory substances in the spent medium.

The results obtained in the present studies have established the nutritional requirement and the optimum conditions for culture of Vigna catiang callus. A basal mineral salts medium containing vitamins, an auxin and a kinin are required for the growth and survival of this tissue. Either White's or Smith's basal salts medium gives the best growth in comparison with other basal media. Several synthetic auxins (2,4,5 trichlorophenoxyacetic acid; 2,4,5 trichlorophenoxy propionic acid, methyl indole acetic acid; β-naphthoxy acetic acid,

α-naphthalene acetic acid and 2,4-dichlorophenoxyacetic acid) were equally effective. Either kinetin or coconut milk showed kinin activity and supported growth, though the latter gave better growth. Both inorganic and organic nitrogen sources (nitrate, yeast extract, coconut milk and casein hydrolysate) were effectively utilized. Sucrose was the best carbon source and the optimum concentration was four to six per cent with kinetin and two per cent with coconut milk. The optimum concentration of coconut milk was 5 to 15 per cent volume of the medium. Tissue growth was markedly stimulated by the addition of both yeast extract and malt extract at 0.1 per cent concentration especially in the presence of coconut milk. The optimum pH was found to be in the range of 5.0 to 7.0. Under these optimum conditions *Vigna catiang* callus cultures have been maintained with periodical sub-cultures for about two years without any diminution in growth.

CHAPTER III

STUDIES ON A.CATIANG TISSUE IN SHAKE FLASKS.

CHAPTER III

SECTION 1

EXPERIMENTAL

STUDIES ON VIGNA CATIANG TISSUE IN SHAKE FLASKS

In addition to establishing conditions for the growth of *Vigna catiang* cultures on semi-solid agar media, the growth of this tissue in liquid media under agitation was then studied. Since the tissue grows as a large, compact mass on agar, the rate of diffusion of nutrients from the agar medium to all the cells may be slow and limit the growth of the tissue. Moreover, the removal of samples of the medium and the determination of the rate of utilization of different constituents of the medium would offer considerable difficulties with agar media. Hence the growth of *V. catiang* was studied in liquid cultures which were agitated on a rotary shaker. Simultaneously the medium was analysed after different periods for nitrogen, phosphorus, iron, sucrose and hydrogen ion concentration. The results of these studies on the growth and metabolism of the tissue are presented in this chapter.

Thirtytwo 250 ml Erlenmeyer flasks, each containing 75 ml of White's basal-yeast extract-malt extract- coconut milk-2,4-dichlorophenoxyacetic acid medium, were inoculated with 400 to 450 mg fresh *V. catiang* tissue and kept on a rotary shaker at 100 to 120 revolutions per minute. At

regular intervals flasks were removed in duplicate and the contents filtered through Whatman No.1 filter paper. The tissue was dried between folds of filter paper and the wet and dry weights were determined as usual. The filtrate was used for analytical determinations after correction for evaporation.

Under these conditions of growth the tissue broke up into fragments and a large number of single cells were also detected in the media under a microscope. However when this tissue suspension was subcultured into fresh media and shaken as before, it was observed that the tissue lost most of its initial friability and clumps composed of a large number of cells were formed and very few or no free floating cells could be observed on microscopic examination.

From Fig.5 it will be seen that the increase in fresh weight of the tissue was about 200 to 250 per cent of the initial inoculum on the seventh day, 400 per cent on the fourteenth day, 1400 per cent on the twentyfirst day and 3000 per cent on the twentysixth day. There was no further increase in growth due possibly to the depletion of some essential nutrient or nutrients in the medium.

The rates of utilization of some of the nutrients at different periods of growth in liquid media were then studied and the results are summarised in Table 16.

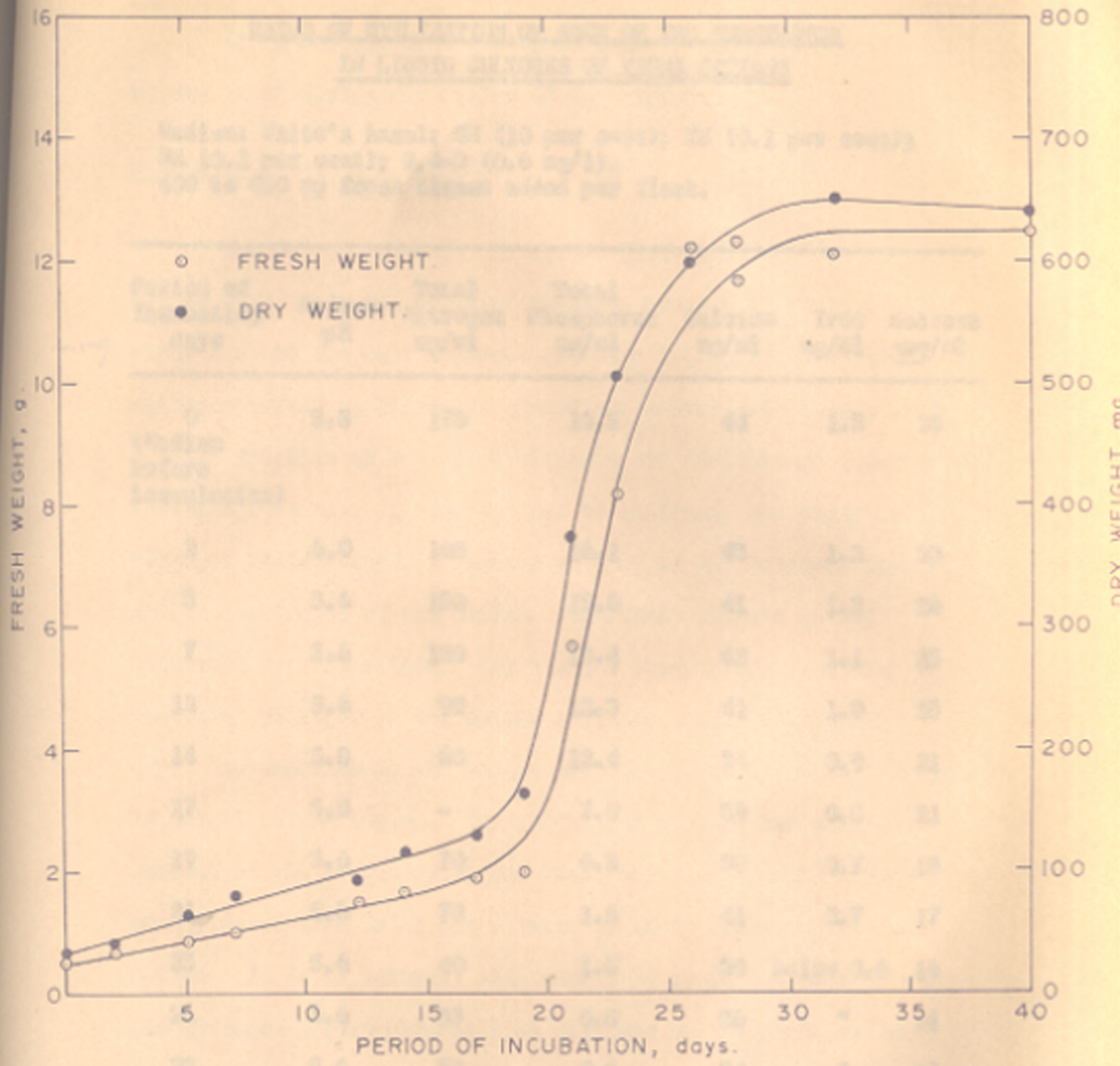


FIG. 5. GROWTH CURVE OF VIGNA CATJANG ON WHITE'S BASAL + COCONUT MILK (10 % V/V) + YEAST EXTRACT (0·1 %) + MALT EXTRACT (0·1%) + 2,4-D (0·6 mg/l). (LIQUID MEDIUM)

TABLE 16

RATES OF UTILIZATION OF SOME OF THE SUBSTRATES IN LIQUID CULTURES OF *MUCUNA CAPIANA*

Medium: White's basal; CH (10 per cent); YE (0.1 per cent); ME (0.1 per cent); 2,4-D (0.6 mg/l).
400 to 450 mg fresh tissue added per flask.

Period of Incubation days	Medium pH	Total Nitrogen ug/ml	Total Phosphorus ug/ml	Calcium ug/ml	Iron ug/ml	Sucrose mg/ml
0 (Medium before inoculation)	5.8	170	16.5	44	1.3	29
2	6.0	160	16.1	43	1.2	28
5	5.6	150	15.6	41	1.2	26
7	5.6	130	13.4	42	1.1	25
12	5.6	90	12.3	41	1.0	25
14	5.8	60	12.4	34	0.9	21
17	5.8	-	7.0	39	0.8	21
19	5.6	70	6.3	39	0.7	18
21	5.6	70	1.6	41	0.7	17
23	5.6	40	1.5	39	below 0.6	16
26	5.6	33	0.8	36	"	14
28	5.6	52	0.8	34	"	14
32	5.6	59	0.8	36	"	14
40	5.6	60	0.8	34	"	13

pH:

It is evident from the table that there is no significant change in pH during the entire 40 day period.

Nitrogen:

The medium initially contained 12.8 mg of Kjeldahl nitrogen (derived from the organic compounds present in coconut milk, yeast extract and malt extract) and only 2.6 mg of inorganic nitrogen (added as nitrate). Since the latter constitutes only a small fraction of the total nitrogen and preliminary experiments indicate that most of the nitrogen is utilized, the study of nitrogen utilization was restricted only to the determination of Kjeldahl nitrogen of the medium. Inorganic nitrate is not estimated by this method. There was a progressive reduction in the total nitrogen in the medium till about the twentysixth day. The decrease in the medium nitrogen paralleled the increase in weight of the tissue. The slight increase in the nitrogen content of the medium after the twentysixth day may be due to autolysis of some cells and release of their contents into the medium. It is of interest that nearly 75 to 80 per cent of all the nitrogen (micro-Kjeldahl) is utilized by the plant cells. It may be noted that except for the set of flasks removed on the fourteenth day the data clearly indicate the progressive utilization of substrates and growth of tissue.

Phosphorus:

The total phosphorus content of the medium (16.5 ug/ml),

which includes inorganic phosphate and also the organic phosphate of coconut milk, yeast and malt extracts, progressively declines to about 0.8 ug/ml on the twentysixth day after which there was no further decrease. The residual phosphorus is possibly either in a non-utilizable form or is the result of release from the cells due to autolysis.

Calcium:

Unlike nitrogen and phosphorus, the calcium content of the medium is apparently in great excess over the requirement for growth and only a small part (between 10 to 20 per cent of total calcium) of the calcium in the medium is utilized over the entire period.

Iron:

The iron content of the medium decreases from 1.3 ug/ml to less than half this value by the twentythird day. Since the quantity of iron was too small to determine at this stage, it is not known whether all the iron in the medium was used up by the tissue.

Sucrose:

For the estimation of sugar in the medium, an aliquot of the medium was hydrolysed with 1N hydrochloric acid in a water bath at 100° for 15 minutes, cooled and then neutralised with 1N sodium hydroxide. The sugars were estimated with

Fehling's reagent according to the procedure of Bertrand and Thomas (1920) and the results were expressed as "sucrose" though sucrose as well as monosaccharides may be present in the medium. The sucrose content of the medium decreases from 29 mg/ml to about 14 mg/ml after 26 days after which there is little or no further utilization. Sucrose like calcium, is therefore, not limiting for growth.

The analytical data indicate that there is progressive utilization of all the medium constituents which were estimated and that sucrose and calcium appeared to be present in adequate quantities whereas nitrogen and phosphorus (and possibly iron) were limiting and the deficiency of one or more probably leads to cessation of growth. The effect of increasing these inorganic constituents was, therefore, studied. The possibility that other nutrients or growth factors are limiting was also examined. Since it is not possible to estimate all the constituents present in yeast extract, etc., additional quantities of yeast and malt extract, 2, 4-dichlorophenoxyacetic acid and kinetin were added after each week. If growth is limited due to any of these constituents being depleted then the addition of these substances should show a growth stimulation. Moreover, these substances when added initially at higher levels to the medium are inhibitory whereas periodic addition of these constituents as they are being continuously utilised would be preferable. Kinetin was added instead of coconut milk since the addition of the latter would

result in undue increase in volume. In the first experiment indicated in Table 17, kinetin, yeast extract, malt extract, 2,4-dichlorophenoxyacetic acid were added at weekly intervals to the tissue growing in shake flasks. The differences observed in the dry weights between the control flasks and the flasks receiving weekly supplements were negligible

The effect of increasing the level of inorganic constituents and adding other compounds such as glutamine, asparagine and inositol which have been found to stimulate growth of plant cells, was then tested.

The composition of the modified medium and the quantities of supplements added at weekly intervals are shown in Table 18. It will be observed that a mere increase in the inorganic salts and supplementation with glutamine, asparagine and inositol did not significantly increase the total tissue (dry weight) per flask above the value of 0.6 gm obtained with unmodified White's basal medium (Table 17). However the addition of yeast extract, malt extract and sucrose resulted in a marked increase in growth which was over twice that of the controls. The addition of glutamine, asparagine and inositol along with these supplements had no additional stimulatory effect whereas supplementation with kinetin and 2,4-dichlorophenoxyacetic acid abolished the effect due to malt extract, yeast extract and sucrose. Additional work

will be necessary to determine whether yeast extract, malt extract or sucrose or a combination of all three is required for enhancing the weight of the tissue. Since it was difficult to obtain large quantities of tissue which are required as inocula for these studies, it was not possible to continue this work on submerged culture of *M. nativus*.

TABLE 17

THE EFFECT OF WEEKLY SUPPLEMENTATION WITH NUTRIENTS ON THE GROWTH OF TISSUE

Medium used: White's basal; Kinetin (1.0 p.p.m.); YE (0.1 per cent); ME (0.1 per cent); 2,4-D(0.6 mg/litre); 400 to 450 mg fresh tissue inoculated into 250 ml flasks containing 75 ml medium.

At weekly intervals 40 ug kinetin, 40 mg yeast extract, 40 mg of malt extract and 40 ug of 2,4-dichlorophenoxyacetic acid were added aseptically into each flask. Control flasks received no replenishments.

Additions	Period of incubation weeks	Wet weight gm/flask	Dry weight gm/flask
None	4	11.5	0.66
YE, ME, 2,4-D	4	11.0	0.65
YE, ME, 2,4-D	5	16.7	0.69
YE, ME, 2,4-D	6	16.0	0.52
YE, ME, 2,4-D	7	15.6	0.39

TABLE 18

EFFECT OF REPLENISHMENTS OF DIFFERENT NUTRIENTS ON VIGNA CATIANG CELLS CULTURED IN SHAKE FLASKS

Medium used: White's basal with KCl (160 mg/l); $NaH_2PO_4.2H_2O$ (320 mg/l); $(NH_4)_2SO_4$ (790 mg/l); $NaNO_3$ (180 mg/l); Ferrous EDTA (5 ml); Sodium molybdate (0.25 mg/l); $CuSO_4.5H_2O$ (0.025 mg/l); $CoCl_2$ (0.025 mg/l); YE and ME (0.1 per cent); glutamine and asparagine (200 mg/l of each); myo-inositol (100 mg/l); 2,4-D (0.6 mg/l); Sucrose (3 per cent); Gibberellic acid (1 mg/l).

YE and ME (40 mg each); sucrose (0.5 gm); kinetin (40 ug); 2,4-D (40 ug); glutamine and asparagine (15 mg each) and myo-inositol (7 mg) were added aseptically at weekly intervals. The replenishments made are specified in the table. Period of incubation: 30 days.

Weekly replenishment	Wet weight gm/flask	Dry weight gm/flask
None	12	0.60
YE, ME and sucrose	20	1.48
YE, ME, sucrose, and 2,4-D	14	0.75
YE, ME, sucrose, glutamine, asparagine, inositol	18	1.42

SECTION 2

DISCUSSION

Suspension cultures have now been derived from a number of different tissues by transfer of tissue fragments to agitated liquid media. An examination of these suspensions has usually shown the presence of free cells, small cell clumps of four to five cells and larger groups of several hundred cells. Moreover, the number of free floating cells has been found to vary with the tissue (Nickell, 1956) depending on its friability and also on the composition of the medium as shown by Torrey and Shigemura (1957). Street and Henshaw (1963) in a review of the work on suspension cultures have pointed out that data regarding the growth rates in suspension cultures are still fragmentary. Torrey et al (1962) working with suspension cultures of Convolvulus root callus observed a marked peak in mitosis after seven days after inoculation, a marked fall in this frequency after two weeks, and no mitoses after three weeks. Though mitoses were rare after fourteen days of culture, the fresh and dry weight of the tissue continued to increase even after this period.

In growth studies with suspension cultures of Vigna catiang derived from hypocotyl callus we find increases

in fresh weight most marked between the 14th and 23rd days with a lower growth rate before 14 days. However mitoses and cell counts were not studied with the cell suspension and hence no conclusions can be drawn as to whether the increases in fresh and dry weight are due to increase in cell number or merely to depositions of starch and cell wall substances. There are many reports on the growth of plant cells in liquid media, (Byrne and Koch, 1962; Tulecke and Nickell, 1960) and several different media have been suggested. The study of the growth of plant cells in shake flasks offers a useful method for determining the rates of utilization of the substances in the medium which may throw light on such important factors as the lag phase and lead to improved media and to better growth. However very few time-course studies on the rates of tissue growth and utilization of medium constituents have been made. Such studies with Vigna radiata suspensions cultures reveal that the utilization of the substrates and parallels the increase in the weight of the tissue and that the nitrogen and phosphorus and possibly iron in the medium are almost completely utilized when growth stops. There is a considerable amount of sucrose and calcium still remaining in the medium at this stage (Table 16).

If growth of the tissue stops because of the depletion of some of the basic nutrients in the medium, then

an addition of different supplements should prolong the growth of the tissue. However surprisingly even when kinetin, yeast extract and malt extract and 2,4-dichlorophenoxyacetic acid were added every week to suspension cultures the overall yield remained unchanged and was the same as in the controls (Table 17).

Reinert and White (1956) considered glutamine at 50 ug/l to be essential for spruce tumor cells. Glutamine could not be replaced by glutamic acid or asparagine. De Torok and Thimann (1961) found asparagine to be indispensable for tumorous and normal tissues of Picea glauca. The presence of myo-inositol in coconut milk has been reported by Pollard et al (1959). Since then inositol has been routinely incorporated in most tissue culture media. Steinhart et al (1961) found that normal tissues of Picea abies grew well in the presence of inositol.

When P. ponderosa callus was grown in a medium containing higher amounts of iron, nitrogen and phosphorus and other salts, as well as glutamine, asparagine and inositol, the growth was not more than that obtained on the standard White's basal medium. On the contrary weekly supplementation of yeast and malt extracts and sucrose resulted in a doubling of the final dry weight (Table 18).

Street and Henshaw (1963) reviewing the earlier work in suspension cultures have stated that in most of the published data only 10 to 30-fold increases could be obtained on a fresh weight basis. With *Vigna catiang* cells grown on a high salt medium, containing glutamine, asparagine and inositol and receiving weekly replenishments of these substances together with yeast extract and malt extract and sucrose, the wet weights showed a 45-fold increase and the dry weights showed a 59-fold increase as compared with the controls which showed a 30 and 24 fold increase in their wet and dry weights respectively. It remains to be determined whether the increase in weight is due to increased cell division or to the deposition of cell material. The observed growth rates are, however, comparable to the best results obtained with other plant tissues and indicate that callus tissue of *V. catiang* is a suitable material for studies which require plant cells capable of rapid growth *in vitro*.

CHAPTER IV

CHEMICAL EXAMINATION OF M.CATIANG TISSUE

CHAPTER IV

EXPERIMENTAL

SECTION 1

CHEMICAL EXAMINATION OF *V. CATIANG* CALLUS TISSUE

Studies on the establishment of callus cultures of *V. catiang* and the optimum conditions for the growth of this tissue were described in previous chapters. The chemical composition of the tissue was then studied and the results of analysis for nitrogen, calcium, phosphorus, carbohydrate, free and bound amino acids and sugars are presented in this chapter.

Nitrogen, Phosphorus and Calcium:

For the identification and estimation of some of the tissue components and constituents, of *V. catiang* callus was grown for three weeks on White's basal-coconut milk-yeast extract-malt extract and 2,4-dichlorophenoxyacetic acid medium. For the estimation of the total nitrogen content the whole tissue was used whereas for the estimation of the alcohol soluble nitrogen, the tissue was extracted with 80 per cent alcohol at 50°. For estimation of calcium the tissue was extracted with dilute acetic acid and calcium estimated as oxalate as described under Materials & Methods. The moisture content of the tissue was determined by drying to constant weight at 110°.

The results are tabulated in Table 19. It will be noted that the moisture content of the tissue is 94 per cent. On a dry weight basis the total nitrogen content of the tissue is 1.6 to 1.7 per cent of which 0.32 per cent is alcohol soluble. If the alcohol insoluble nitrogen is assumed to be protein nitrogen, the protein content (N x 6.25) of the tissue is 8.85 per cent on a dry weight basis.

TABLE 19

ANALYSIS OF *VICIA CAPIANA* CALLUS TISSUE

	mg/gm fresh tissue
Dry weight	61.0
Total nitrogen	1.06
Alcohol soluble nitrogen	0.19
Calcium	0.26
Total phosphorus	0.17

Free and bound amino acids:

The free amino acids present in the callus tissue and the amino acids of the tissue protein were then determined. The free and bound amino acids of the hypocotyls of *V. antiana* seedlings were also determined at the same time, so that the amino acid composition of the callus tissue and

the normal tissue could be compared. The seedlings were obtained 6 days after germination and the hypocotyls were removed.

The callus tissue and the hypocotyls were separately weighed and extracted by grinding under hot 80 per cent alcohol with acid-washed sand. The extraction was repeated four times with 80 per cent alcohol at 50° and the extracts of each tissue were combined. The total ethanolic extracts were then separately passed through 1 x 5 cm bed of Dowex 50 x 8 (H form) 200-400 mesh to remove the free amino acids and amides. The columns were washed with deionised water. The unadsorbed material and washings were combined and used for the identification of sugars which are not retained on the column. For the detection and estimation of the protein-amino acids, the alcohol insoluble residues were dried in vacuo over phosphorus pentoxide in a Fischer dryer at 110°. Aliquots of the residues were hydrolysed separately with 6N hydrochloric acid at 110° for 18 hours in evacuated sealed tubes. The hydrolysates were taken to dryness several times in a vacuum desiccator over concentrated sulphuric acid and potassium hydroxide and then dissolved in isopropyl alcohol-water and adsorbed on Dowex 50 H^+ columns as described above. The free and bound amino acids were eluted from the Dowex-50 columns by the method of Plaisted (1958) and were analysed quantitatively by the two

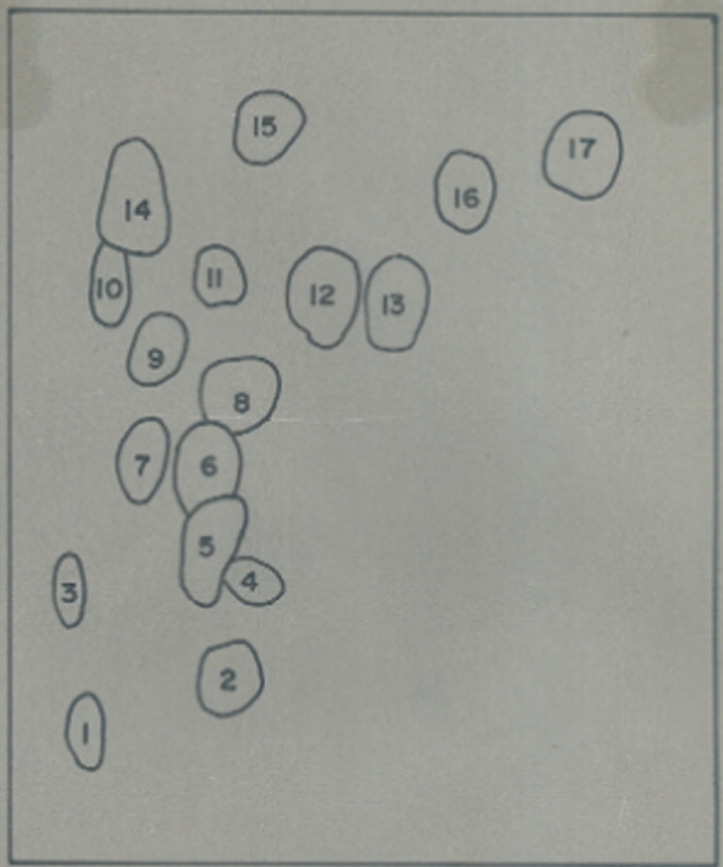


PLATE 3: Two dimensional chromatogram of a standard mixture of amino acids.

1. Cys SO_3H; 2. Asp. 3. Cys. 4. Glu. 5. Ser. 6. Gly. 7. Asp. NH_2; 8. Thr. 9. Lys. 10. Arg. 11. Hy. Prol. 12. Ala. 13. Tyr. 14. Hist. 15. Prol. 16. Val+Meth. 17. Phe., Ileu., Leu.

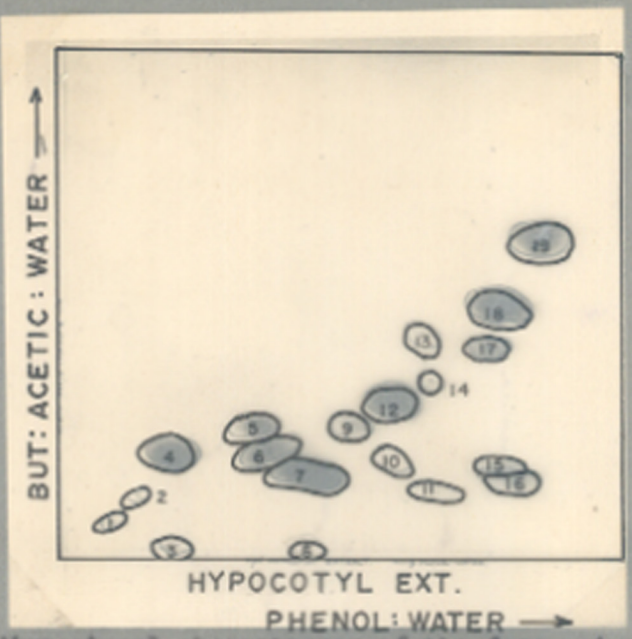


PLATE 4: Two dimensional chromatogram of the free amino acids of Vigna sinensis hypocotyl.
1. Cys.SO_3H. 2. Uniden. 3. Uniden. 4. Asp. 5. Glu. 6. Ser. 7. Asp NH_2 8. Cyst. 9. Thr. 10. GluNH_2 11. Lys. 12. Ala. 13. Tyr. 14. Uniden. 15. Hist. 16. Arg. 17. Val. 18. Meth. 19. Phe., Ileu., Leu.

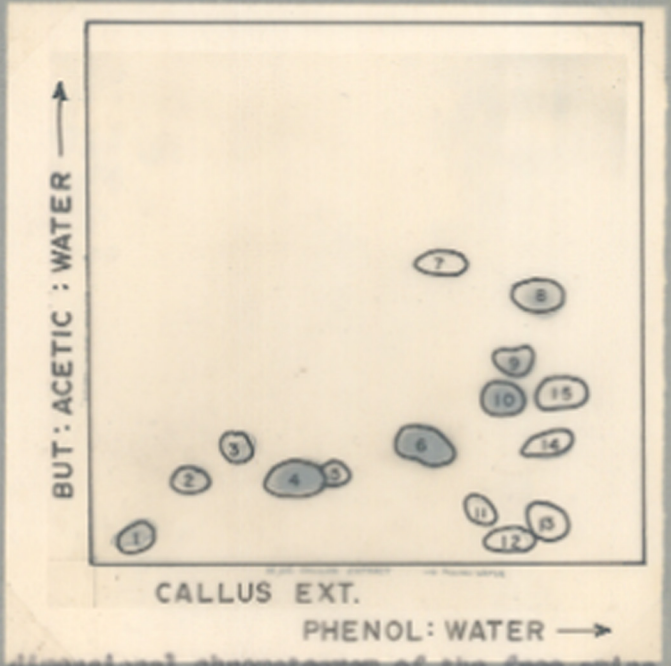


PLATE 5: Two dimensional chromatogram of the free amino acids of Vigna sinensis callus.

1. Cys.SO_3H 2. Asp. 3. Glu. 4. Gly. 5. Uniden. 6. Ala. 7. Unidem. 8. Phe., Ileu., Leu., 9. Meth. 10. Val. 11. Lys. 12. Arg. 13. Hist. 14. Prol. 15. Unidem.

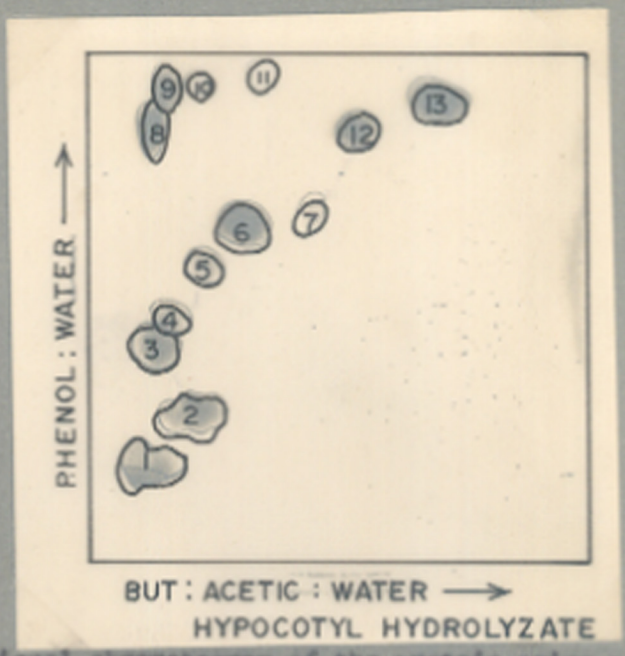


PLATE 6: Two dimensional chromatogram of the protein amino acids of Vigna radiata hypocotyls.

1. Asp. 2. Glu. 3. Ser. 4. Gly., 5. Thr. 6. Ala. 7. Tyr.
8. Arg. 9. Hist. 10. Hy.Prol. 11. Prol. 12. Val. 13. Phe., Ileu., Leu.

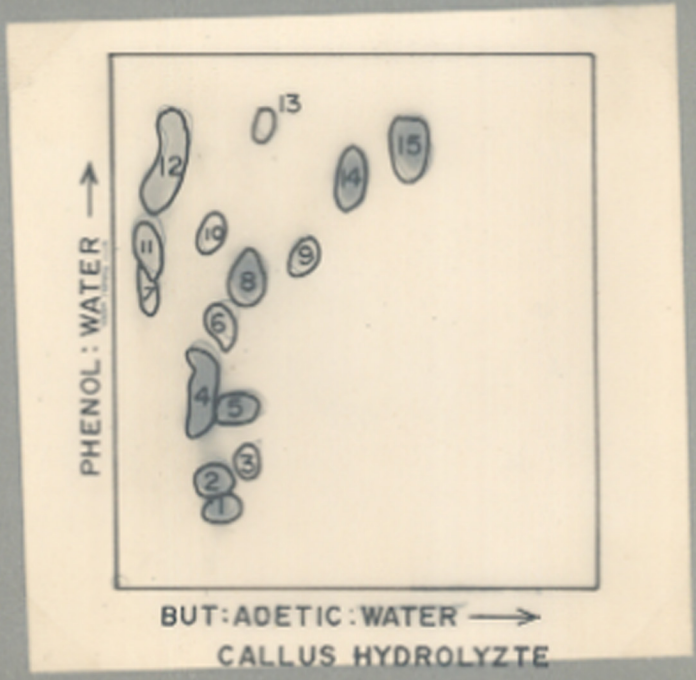


PLATE 7: Two dimensional chromatogram of the protein amino acids of Vigna radiata callus.

1. Unidea. 2. Asp. 3. Unidea. 4. Ser. 5. Glu. 6. Gly. 7. Tyr.
8. Thr. 9. Ala. 10. Hy.Prol. 11. Lys. 12. Arg., Hist. 13. Prol.
14. Val. 15. Phen., Ileu., Leu.

dimensional paper chromatographic procedure of Porter et al (1957). The amino acids were identified by comparison with chromatographic runs of standard amino acids.

The free amino acid composition of the callus and hypocotyls is shows in Table 20. *L. sativum* hypocotyls and hypocotyl callus cultures showed differences in the nature and contents of their free amino acids. Serine, asparagine, threonine and tyrosine were present in the hypocotyls but were not detected in the callus tissue whereas glycine was detected in the callus tissue but not in the hypocotyls. On a fresh weight basis hypocotyls contained a greater amount of free amino acids. The aspartic acid and asparagine contents in the hypocotyls were very high and accounted for roughly 25 per cent of the total amino acids on a weight basis while only aspartic acid is present in the callus and represents only 5 per cent of the total amino acids. Since phenylalanine, isoleucine and leucine in the standard amino acid run appeared as a single spot the corresponding spot in the extracts was assumed to be a combination of the three.

Three unidentified ninhydrin positive spots were also detected in chromatograms of callus extract, having R_f values of 0.14, 0.172 and 0.50 in butanol:acetic acid: water and 0.41, 0.73 and 0.603 in phenol:ammonia:water respectively. Three unidentified ninhydrin positive spots were also detected in the

hypocotyl extract/ one having an R_f of 0.281 and 0.634 in butanol:acetic acid:water and phenol:ammonia:water respectively.

The amino acid composition of the tissue proteins is shown in Table 21. The values are expressed in terms of amino acid nitrogen as percentage of total nitrogen determined.

From Table 21 it can be seen that the same amino acids are present in both callus and hypocotyl proteins except for histidine which is very low in the callus. The hypocotyl proteins are markedly richer than the callus in aspartic acid and glycine whereas the callus tissue proteins are richer in arginine.

Carbohydrates:

The sugars in the callus extract were isolated from the fraction not adsorbed on Dowex-50 as indicated above. This fraction and washings from the column were passed through a 1 x 10 cm bed Dowex 1x10 (200-400 mesh) (acetate form) to remove the non-volatile acids. The fraction passing through the Dowex-1 column which contained sugars was taken to dryness. The dry residue was dissolved in a small volume of water, shaken with chloroform to remove pigments and the aqueous layer was kept for sugar analysis. The identification of the sugars was carried out by one dimensional ascending paper chromatography on Whatman No.4 filter paper. Runs were carried out using two different solvent systems: butanol:acetic acid:water (4:1:5, v/v) and phenol saturated

TABLE 20

FREE AMINO ACIDS OF VIGNA CATJANG CALLUS & SEEDLING HYPOCOTYLS

Hypocotyls were cut from 6 day old germinating seedling. Callus was obtained from 21 day old tissue grown on White's basal, coconut milk, yeast extract, malt extract and 2,4-dichlorophenoxyacetic acid medium.

	Amino acid	Hypocotyl ug/gm wet tissue	Callus ug/gm wet tissue
1	Cysteic acid	30	32
2	Aspartic acid	56	10
3	Glutamic acid	15	4
4	Glycine	not detected	13
5	Serine	16	not detected
6	Asparagine	73	not detected
7	Lysine	8	1
8	Arginine	33	4
9	Alanine	50	32
10	Threonine	17	not detected
11	Tyrosin	5	not detected
12	Valine	17	19
13	Methionine	50	12
14	Histidine	7	10
15	Proline	trace	14
16	Hydroxyproline	not detected	not detected
17	Phenylalanine		
18	Isoleucine	50	17
19	Leucine		

TABLE 21

PROTEIN AMINO ACIDS OF V.CATIANG CALLUS & SEEDLING HYPOCOTYLS

Hypocotyls were excised from 6 day old germinated seedlings. Callus tissue was obtained from 21 day old tissue grown on White's basal-coconut milk-yeast extract-malt extract-2,4-dichlorophenoxyacetic acid medium.

	Amino acid	Hypocotyl tissue Amino acid N as % of total N	Callus tissue Amino acid N as % of total N
1	Aspartic acid	8.7	3.2
2	Glutamic acid	5.5	6.6
3	Serine	7.9	11.0
4	Glycine	5.4	1.8
5	Threonine	3.4	4.6
6	Alanine	10.7	10.0
7	Tyrosine	1.2	1.8
8	Valine	5.6	5.8
9	Histidine	3.2	trace
10	Arginine	8.0	15.6
11	Lysine	16.8	14.7
12	Proline	4.7	6.3
13	Hydroxyproline	1.8	2.8
14	Phenylalanine		
15	Leucine	17.8	16.0
16	Isoleucine		

with water. Guide strips of known sugars and of the tissue extracts were developed with 0.2 per cent naphtheresorcinol in ethanol and phosphoric acid (9:1 v/v) at 90° for 5 minutes according to the method of Bryson and Mitchell (1951). 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 millilitres 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 ten minutes and the optical density was read at 640 mμ in a Unicam SP 600 spectrophotometer.

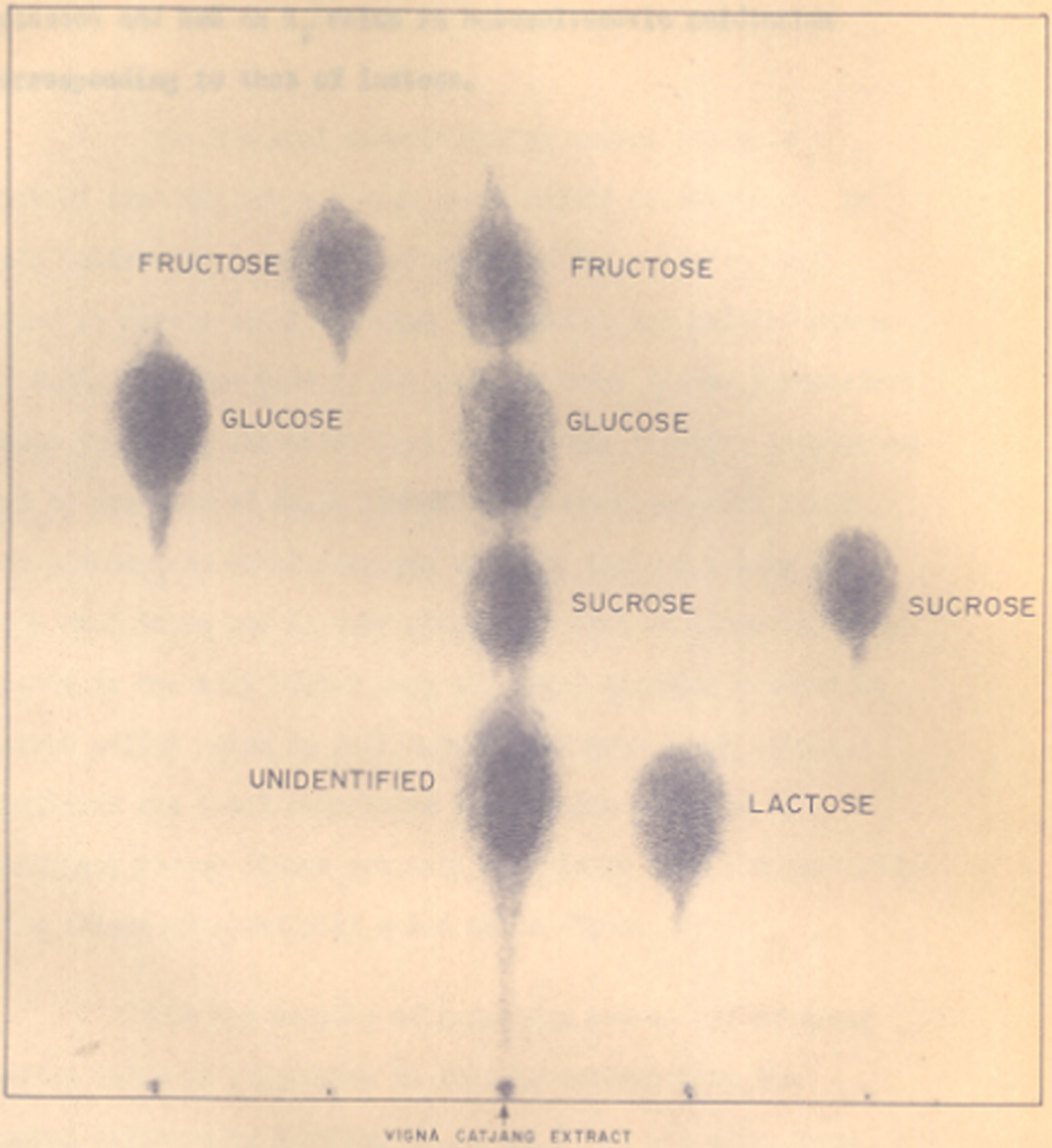
Sucrose, fructose and glucose were identified unambigiously in extracts of *L. catiang* callus and the quantities are shown in Table 22. The identity of these

TABLE 22

CARBOHYDRATES OF *KIGNA CATIANG* CALLUS CULTURES

	mg/gm fresh tissue
Glucose	0.65
Sucrose	0.82
Fructose	0.76

sugars was also confirmed by preparation of the osazones and observing the characteristic osazone crystals under the microscope. The three sugars are present in nearly equal amounts in the tissue. An unidentified sugar which reacted



SOLVENT SYSTEM: BUTANOL: ACETIC: WATER.
(4:1:5 V/V)

FIG. 6. IDENTIFICATION OF SUGARS PRESENT IN EXTRACTS OF VIGNA CATJANG CALLUS.

with the naphthoresorcinol-phosphoric acid reagent was also detected and had an R_f value in butanol:acetic acid:water corresponding to that of lactose.

SECTION 2

DISCUSSION

The chemical composition of callus cultures derived from different plants shows marked differences. In this Laboratory (unpublished) opium callus cultures were found to have a total nitrogen, phosphorus and calcium content of 2.85, 0.26 and 0.56 mg per gram of fresh tissue respectively. Maize callus on the other hand has a total nitrogen content of 2.5 mg per gram of fresh tissue. In comparison with these two tissues the total nitrogen of *Vigna catiang* callus is very much lower and is only about half that of these tissues. But it is markedly higher than the total nitrogen content of carrot callus which is only 0.69 mg per gram fresh weight. Similarly the total phosphorus and calcium contents of *V. catiang* tissue tissue are very much lower than the corresponding values obtained with opium cells (Table 19).

The dry weights of *V. catiang* tissue and of other callus cultures maintained in this Laboratory also show marked differences (150 mg, 72 mg and 57 mg per gram fresh tissue respectively for rose bud, opium and carrot). *V. catiang* tissue has a relatively high water content.

A comparison of the chemical composition of parts of intact plants with that of the corresponding callus tissue has been made for a few plants and marked differences have been observed. Bove *et al* in 1957 found that tissue cultures

of citrus contained more γ-amino butyric acid and proline than did the intact plant and that the level of potassium in the culture medium strongly influenced the amounts of free amino acids in the tissue cultures. Similarly Steward and Pollard in 1958 observed that in potato, the protein of cells in tissue culture was richer in hydroxyproline and basic amino acids than the protein of mature potato tuber cells. In the case of Agave, Weinstein et al (1959) observed that the protein showed a greater amount of hydroxyproline in tissue culture than the leaf tissue. Free hydroxyproline was also shown to be present in the callus but not in the leaves. Tulecke et al (1962) found marked differences in the amounts and kinds of protein amino acids of the tissue culture and germinated pollen of Ginkgo biloba. L. catiang hypocotyls and callus culture have a similar protein amino acid content though the hypocotyls are richer in aspartic acid, glycine and histidine whereas the callus tissue is richer in arginine (Table 21).

The free amino acids of L. catiang callus cultures and hypocotyls also showed marked differences. Serine, asparagine, threonine and tyrosine were present in the hypocotyls but not in the callus cultures, whereas proline was detected in the callus cultures but not in the hypocotyls (Table 20).

Weinstein et al (1959) reported that tissue cultures of Agave leaves contained sucrose, glucose and

fructose the main sugar being fructose followed by glucose and sucrose. They also obtained some unidentified spots in their paper chromatograms one of which was later identified as raffinose. Weinstein et al (1962) found sucrose to be the dominant sugar and glucose and fructose to be present in smaller amounts in tissue cultures of scarlet rose. The contents of sucrose, fructose and glucose were very much lower in Vigna catiang callus cultures than in the callus cultures of Agave and scarlet rose (Weinstein et al. 1959, 1962). These three sugars were found to be present in nearly equal amounts in Vigna catiang callus (Table 22).

CHAPTER V

RESPIRATION STUDIES ON V.CATIANG CALLUS TISSUE

CHAPTER V

SECTION 1

RESPIRATION STUDIES ON *MIGNA CATIANG* CALLUS TISSUES

The oxidation of several different substrates by *Migna catiang* tissue grown *in vitro* was studied. Preliminary experiments indicated that the tissue contained considerable amounts of sugars and several endogenous substrates and hence the oxygen uptake was considerable even in the absence of added substrates. The addition of glucose, fructose and sucrose for instance caused very little increase in oxygen uptake in the presence of the plant cells since the cells contained reserves of carbohydrate which were readily oxidisable. Starvation of the cells on sugar deficient media for even three to four days did not cause an appreciable reduction in the endogenous oxygen uptake. Hence the studies reported in this section will be restricted mainly only to those substrates which caused a marked stimulation of oxygen uptake and to a few related compounds. The effect of some inhibitors on oxygen uptake was also tested. The variation in the rates of oxygen uptake with acetate by the plant cells grown for different periods was also studied since there was a marked difference in the rate of acetate oxidation by cells in the earlier and later stages of growth.

Vigna catiang tissue was grown on semi-solid media in test tubes on White's basal medium containing coconut milk, yeast extract, malt extract and 2,4-dichlorophenoxyacetic acid. The tissue was grown for different periods and was carefully removed from the tube so that the adhering agar from the medium was separated from the plant cells. Aliquots of 0.5 gm fresh tissue were weighed and transferred by means of a forceps to the main compartment of the Warburg flasks. The rate of oxygen uptake was determined by conventional Warburg manometry at 30°. The substrates tested were in the side bulb and the centre well contained 0.2 ml of 20 per cent (w/v) potassium hydroxide and a strip of filter paper. The incubation mixture contained in addition to 0.5 gm of fresh cells, 100 umoles of phosphate buffer pH 6, substrate and distilled water to give a final volume of 3 ml. The gas phase was air. After equilibration for 10 min at 30° the substrates were tipped in from the side bulb and the oxygen uptake was noted every 10 min for a period of three to four hours. The rate of oxygen uptake was linear during this period. The average rate of oxygen uptake was calculated from the readings obtained during the first three or four hours. The results are expressed as micromoles of oxygen taken up per hour per mg of tissue nitrogen at 30°. The nitrogen content of the tissue was determined by the micro-Kjeldahl method and was found to be 1.06 mg nitrogen per gm of fresh weight of tissue.

The blanks were run in all cases in the absence of any added substrate. This endogenous oxygen uptake was subtracted from the oxygen uptake in the presence of each substrate and net oxygen uptake for each particular substrate was expressed as QO_2 in terms of micromoles of oxygen consumed per hour per mg tissue nitrogen. The net oxygen uptake was expressed as a negative value in accordance with the usual convention whereas a positive value obtained in a few cases indicated oxygen uptake less than that of blank and probably represents a slight inhibition of the endogenous oxygen uptake by a particular substrate.

In the first set of experiments the oxidation of several citric acid cycle intermediates was studied. The rates of oxidation of pyruvate, succinate, fumarate, acetate, citrate, isocitrate, malate and α-ketoglutarate are shown in Tables 23 and 24. In addition the oxygen uptake with glyoxylate and ascorbate was also determined using 18 day old tissue. It will be observed that compared to the endogenous uptake there was marked oxygen uptake only with acetate and to a smaller extent with fumarate with 16 day old tissue. With 18 day old tissue there was stimulation of oxygen uptake only with acetate, α-ketoglutarate and to a lesser extent with isocitrate. Glyoxylate also increased the rate of oxygen consumption. A very great increase in oxygen uptake was, however, observed only with ascorbate. The pH of the blank and of the experimental was 4.7 with ascorbate owing

TABLE 23

OXIDATION OF CITRIC ACID CYCLE INTERMEDIATES BY *A.CATIANG* CALLUS

16 day old tissue was used for these studies. The test system consisted of 0.5 gm fresh weight of tissue, 100 umoles potassium phosphate and substrate in a final volume of 3.0 ml, pH 6.0. Gas phase - air; KOH in centre well. Temperature 30°. The micromoles of substrate used are shown in parenthesis: Pyruvate (60); Succinate (30); Acetate (20); Fumarate (30).

Time hours	Microliters of oxygen utilized per hour per mg tissue nitrogen						
	Endogenous	Pyruvate	Pyruvate + succinate	Succinate	Succinate + Acetate	Fumarate	Acetate
First	147	143	160	147	187	125	204
Second	147	149	156	147	187	129	196
Third	151	146	160	151	191	125	212
Average	148	146	159	148	188	126	204
QO_2 corrected for blank		2	-11	0	-40	-22	-56

TABLE 24

OXIDATION OF KREB'S CYCLE ACIDS AND ASCORBATE AND GLYOXYLATE BY *VIGNA CATIANG* TISSUE

13 day old tissue was used for these experiments. The test system consisted of 0.5 gm fresh tissue, 100 umoles potassium phosphate and substrate, in a final volume of 3.0 ml, pH 6.0. With ascorbate pH was 4.7. Gas phase - air; KOH in centre well. Temperature 30°. The micromoles of substrate used are shown in parenthesis: Citrate (30), Isocitrate (30), Malate (30), Acetate (20), α-Ketoglutarate (30); Ascorbate (50), Glyoxylate (30).

Time hours	Microliters of oxygen utilized per hour per mg tissue nitrogen							
	Endogenous	Citrate	Isocitrate	Malate	Acetate	α-Keto-glutarate	Ascorbate	Glyoxylate
First	133	129	149	136	190	162	341	161
Second	158	140	166	138	212	182	412	190
Third	154	144	174	136	196	193	370	184
Average	151	139	162	138	180	196	368	183
QO_2 corrected for blank		12	-11	13	-28	-45	-217	-32

to the instability of this vitamin at higher pHs.

A lack of marked stimulation of oxygen uptake with several of the citric acid cycle intermediates may, however, be due to lack of permeability of the cells to these substrates or to the presence of endogenous reserves. The oxidation of acetate was tested both directly and with succinate since a citric acid cycle member is generally required for sparking the oxidation of acetate or pyruvate. But in these experiments no additional enhancement in oxygen uptake was observed when both succinate and acetate were used. Among other substrates tested alcohol was found to be oxidised at an appreciable rate (Table 25) and the rate of oxidation was nearly the same at pH 6.0 and pH 8.0.

TABLE 25

OXIDATION OF ETHANOL BY *MYNA CATIANA* TISSUE

The test system was the same as in Table 23, except that the pH was adjusted to 6.0 or 8.0 as shown in the table. 150 micromoles alcohol was taken. 30 day old tissue was used for this experiment.

Time hours	Microliters of oxygen utilized per hour per mg tissue nitrogen		
	Endogenous pH 6.0	pH 6.0 150 umoles alcohol	pH 8.0 150 umoles alcohol
First	61	104	121
Second	53	97	93
Third	68	120	121
Average	61	107	113
Q_{O_2} corrected for blank		-46	-53

When the above experiments with succinate and acetate were repeated (Table 26) with 22 day old tissue, it was observed that in contrast to the experiments with 16 day old tissue succinate was oxidised at a measurable rate whereas there was no stimulation in oxygen uptake with acetate. Other experiments with 30 day old

TABLE 26

OXIDATION OF ACETATE, GLYOXYLATE AND SUCCINATE BY *VIGNA CATIANG* TISSUE

22 day old tissue was used in these experiments. The test system consisted of 0.5 gm fresh tissue, 100 umoles potassium phosphate and substrate, in a final volume of 3.0 ml, pH 6.0. Gas phase - air; KOH in centre well. Temperature 30°. The micromoles of substrate used are shown in parenthesis: Acetate (20); Glyoxylate (30); Succinate (30).

Time hour	Microliters of oxygen utilized per hour per mg tissue nitrogen			
	Endogenous	Acetate	Glyoxylate	Succinate
First	133	119	160	165
Second	140	136	153	199
Third	147	126	142	159
Fourth	164	146	167	159
Average	146	132	155	168
QO_2 corrected for blank		14	-9	-22

tissue also showed that the capacity for oxidation of acetate was much less in the older tissue whereas 16 or 18 day old tissue oxidised acetate at a much greater rate. The effect of glyoxylate

was also tested separately or together with acetate but there was no increase in oxygen uptake in the presence of both the substrates.

The variation in the rate of acetate oxidation suggested that the glyoxylate pathway may occur in this tissue in the earlier stages of growth. Attempts were, therefore, made to test for isocitratase, an important enzyme of the glyoxylate pathway, which cleaves isocitrate to succinate and glyoxylate. The results were inconclusive because of the very high blanks. It will, therefore, be necessary to obtain large quantities of tissue and carry out a preliminary purification before a test for the individual enzymes of the glyoxylate pathway can be undertaken.

The effect of variation in pH and the addition of 2,4-dichlorophenoxyacetic acid, kinetin, coconut milk and the salts of the White's basal medium was then studied but there was no significant variation in oxygen uptake in the presence of these supplements. Hence these results are not reported separately.

The effect of several inhibitors is reported in Table 27. The results in this table are expressed as microlitres of oxygen taken up per hour per mg tissue nitrogen without correction for the endogenous blank so as to facilitate a comparison of the endogenous uptake with the oxygen uptake with succinate and

TABLE XX

THE EFFECT OF INHIBITORS ON THE RESPIRATION OF *VIGNA CATIANG* TISSUE

23 day old tissue was used in these experiments. The test system consisted of 0.5 gm fresh tissue, 100 umoles potassium phosphate and substrate in a final volume of 3.0 ml, pH 6.0. Gas phase - air; KOH in centre well. Temperature 30°. The micromoles of inhibitor used are shown in parenthesis: 30 micromoles succinate was added to all flasks except control. Sodium azide (10); Malonate (10); 8-Hydroxyquinoline (10); Potassium cyanide (10).

Time hour	Microliters of oxygen utilized per hour per mg tissue nitrogen						
	Control	Succinate+ sodium azide	Succinate+ malonate	Succinate+ sodium fluoride	Succinate+ 8-Hydroxyquinoline	Succinate+ potassium cyanide	Succinate
First	125	113	129	113	121	64	159
Second	113	96	125	96	87	90	162
Third	133	109	140	111	121	86	170
Average	124	106	131	107	109	76	164

succinate + inhibitor). 23-day-old tissue was used for these studies in the presence of succinate. It will be seen from the table that malonate showed very little inhibition. Since malonate is an inhibitor of succinic dehydrogenase, the absence of inhibition suggests that the cells are not probably permeable to malonate. Marked inhibition was observed with 8-hydroxy-quinoline and sodium fluoride, sodium azide and potassium cyanide. Oxygen uptake with these substrates was also less than the endogenous respiration indicating that even the endogenous oxygen uptake is inhibited by these compounds. Sodium fluoride inhibits glycolysis at the enolase stage whereas sodium azide and cyanide are inhibitors of the respiratory enzymes especially at the terminal cytochrome oxidase step. It is however of interest that even in the presence of 0.003M cyanide concentration the oxygen uptake was not completely inhibited and more than 50 per cent of the endogenous uptake is unaffected by this inhibitor. Since cytochrome oxidase is almost completely inhibited at this cyanide concentration, this experiment suggests that the endogenous oxygen uptake is partly due to the cytochrome pathway and partly due to a cyanide insensitive pathway.

SECTION 2

DISCUSSION

Tamaoki *et al* (1960, 1961) reported that mitochondria from plant tissues grown *in vitro* show a capacity for oxidation of citric acid cycle members and also for oxidative phosphorylation. They also reported a high rate of ascorbic acid oxidation compared to the oxidation of reduced diphosphopyridine nucleotide. The tissue available was insufficient for the isolation of mitochondria from *Vigna catiang*, though this would have been of value in eliminating the high substrate blanks. However, the results reported in this section show that the *Vigna catiang* cells have the capacity to oxidise acetate, succinate, fumarate, α-ketoglutarate, glyoxylate, alcohol and ascorbate. The stimulation of oxygen uptake by α-ketoglutarate and the lower activity with isocitrate and the absence of pyruvate oxidation indicate that these differences are probably due to differences in permeability. The high rate of ascorbic acid oxidation is in agreement with that reported by Tamaoki *et al* (1960) for mitochondria obtained from plant tissues grown *in vitro*. The variation in the acetate oxidation was particularly of interest and suggests that there is variation in the ability to oxidise this substrate as the age of the tissue varies. These experiments, however, require to be extended further and preferably by tracer studies, since the results can also be due to changes in

permeability. The experiments with the inhibitors indicate that the respiration is sensitive to fluoride, azide, cyanide and 8-hydroxyquinoline but not to malonate. The effect of 8-hydroxyquinoline is probably due to the requirement of a trace amount of metal, but further studies will be required to determine which metal or metals are being chelated by this compound. Complete suppression of the increase of oxygen uptake due to succinate and the lowering of the uptake even below the endogenous value in the presence of azide and cyanide indicates that succinate is oxidized by the cytochrome route since these two inhibitors act on cytochrome oxidase. Part of the endogenous respiration is also sensitive to these inhibitors, but part of the endogenous respiration is probably due to a non-cytochrome pathway which is insensitive to cyanide.

SUMMARY AND CONCLUSIONS

SUMMARY & CONCLUSIONS

Tissue culture techniques have wide application in several fields of experimental biology, particularly in the understanding of cell physiology and pathology, in the comparative study of normal and tumor cells and of viruses. Extensive studies have, therefore, been carried out in recent years to establish cultures of a wide range of animal and plant tissues and to study their nutritional requirements and metabolism.

The present study deals with the isolation for the first time of callus cultures from hypocotyls of *Vigna sinensis* seedlings, the nutritional requirements of the callus cultures on solid media, the utilisation of nutrients in the medium by cultures grown in shake flasks, chemical analysis of the tissue and the respiration of the callus tissue.

For the initiation of callus formation from the seedling hypocotyls, White's basal medium supplemented with coconut milk, yeast extract and malt extract was found to give good results. The presence of an auxin is not required possibly due to adequate amounts of auxin carried over in the explants themselves.

However, on subculture growth occurred only when an auxin, such as naphthalene acetic acid or 2,4-dichlorophenoxyacetic acid and a kinin such as coconut milk or kinetin,

are added to White's medium. In the absence of either auxin or kinin the tissue fails to survive. The addition of both yeast extract and malt extract markedly stimulated growth, especially in the presence of coconut milk.

Of the several different basal media tested Smith's and White's mineral salt media were found to be superior to other media. Studies on the effect of a number of different organic and inorganic nitrogen sources on growth indicated that yeast extract, casein hydrolysate or coconut milk as the sole nitrogen source give excellent growth of the tissue. Nitrates are superior to ammonium compounds as sources of nitrogen.

Studies of the effect of different carbohydrate sources on cell growth showed that sucrose was the best carbohydrate source followed by lactose, glucose and maltose. The optimum concentration of sucrose in a fully supplemented medium containing yeast extract, malt extract, 2, 4-dichlorophenoxyacetic acid and kinetin was 4 to 6 per cent, whereas in a similar medium containing coconut milk instead of kinetin, the optimum sucrose concentration was 2 per cent. Glycerol also sustained appreciable growth as the sole carbohydrate source whereas sorbitol and ethanol were found to be ineffective. Several long chain fatty acids and a number of plant extracts such as lemon juice, Vigna catiang extract, rice washing etc. were tested for kinin activity and for growth stimulation in the presence of coconut milk, but none of these supplements showed

kinin activity or stimulation of growth.

The effect of a number of different auxins on the growth of Vigna catiang was tested. 2,4,5-Trichlorophenoxypropionic acid, methylindole-3-acetic acid or 2,4,5-trichlorophenoxyacetic acid, β-naphthoxyacetic acid, indolepropionic acid, 2,4-dichlorophenoxyacetic acid and naphthalene acetic acid were found to be active in maintaining tissue growth whereas indole acetic acid, 2-benzothiazolyloxyacetic acid were inactive.

Vigna catiang callus showed a broad pH optimum between 5.0 and 7.0. Increase in the partial pressure of oxygen or carbon dioxide was found to have no effect compared to the growth of the tissue in air on agar medium whereas growth on liquid medium was enhanced by passage of oxygen instead of air.

Growth curve studies with Vigna catiang tissue indicate an initial lag period of about 10 days, rapid growth till about the end of the fourth week followed by a cessation of growth. When Vigna catiang callus tissues are grown in liquid media on a rotary shaker, the initial growth rate is slow till the 14th day and rapid from the 14th to the 23rd day.

Studies on the utilization of some of the nutrients by the tissue in shake flasks reveal rapid and almost complete

utilization of the organic nitrogen, phosphorus and possibly iron in the medium when growth of the tissue stops. When the tissue was grown in a modified White's medium under submerged conditions a 200 per cent increase in the final dry weight of the tissue over the controls was obtained by weekly supplementation with yeast extract, malt extract and sucrose.

Vigna catiang callus contains 94 per cent moisture. The nitrogen, phosphorus and calcium contents per gram of fresh tissue were found to be 1.06, 0.17 and 0.26 mg respectively.

The chief differences between the free amino acids of Vigna catiang callus tissue and hypocotyl tissue of V. catiang were the presence of proline in the former and of serine, asparagine, threonine and tyrosine in the latter, whereas the other amino acids were the same in both. The protein amino acids of the callus and hypocotyls differ only in the higher arginine and lower aspartic acid and glycine of the callus tissue in comparison with the hypocotyls.

The main carbohydrates of the callus tissue were sucrose, fructose and glucose which were in nearly equal amounts.

Vigna catiang callus was found to oxidize acetate, succinate, glyoxylate, alcohol, α-ketoglutarate and ascorbate. Studies on the effect of inhibitors indicate that the respiration is sensitive to fluoride, azide, cyanide and 8-hydroxyquinoline but not to malonate.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ball, E. (1950) Growth 14, 293.

Ball, E. (1953) Bull. Torrey Bot. Club 80, 409.

Ball, E. (1955) Ann. Biol. 31, 81.

Bergmann, L. (1959) Trans. N. Y. Acad. Sc. 21, 227.

Bergmann, L. (1960) Jour. Gen. Physiol. 43, 841.

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

Bobilioff-Preisser, W. (1917) Beih. bot. Zbl. 33, 248.

Bove, J., Bove, C. and Raveux, G., (1957) Rev. Gen. Bot. 64, 572.

Borger, H. (1926) Arch. exp. Zellforsch. 2, 123.

Braun, A. C. and White, P. R. (1943) Phytopathol. 33, 85.

Braun, A. C. and Naf, U. (1954) Proc. Amer. Soc. Expt. Biol. & Med. 86, 212.

Braun, A. C. and Stonier, T. (1958) Protoplasmologia 10, 1.

Brown, R. (1833) Trans. Linnean Soc. 16, 685.

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

Burkholder, P. R. & Nickell, L. G. (1949) Bot. Gaz. 110, 426.

Byrne, A. F. & Koch, R. B. (1962) Science, 135, 215.

Capito, L. de (1952) C. R. Soc. Biol. 146, 363.

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

Czeck, H. (1927) Arch. f. exp. Zellf. 3, 176.

David, S. B. & Street, H. E. (1961) Prof. S. P. Agharkar Commem. Vol. p. 97.

Davies, E. A. (1949a) Proc. Amer. Hort. Sci. 53, 233.

Duhamet, L. (1950) Compt. rend. soc. biol. 144, 59.

Dujardin, F. (1837) Ann.sci.nat.Zool. 4, 343.

Ferguson, J. D. (1963) Physiol. Plant. 16, 585.

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

Frank, E. M., Riker, A. J. & Dye, S. L. (1951) Plant Physiol. 26, 258.

French, D. I. and Gibson, M. R. (1957) J.Pharm.Sc. 46, 151.

Ganapathy, P. S., Hildebrandt, A. C. & Riker, A. J. (1964) Amer. Jour. Bot. 51, 669.

Gautheret, R. J. (1933) C. R. Acad. Sci. Paris, 196, 638.

Gautheret, R. J. (1934) C. R. Acad. Sci. Paris, 198, 2195.

Gautheret, R. J. (1939) C. R. Acad. Sci. Paris, 210, 632.

Gautheret, R. J. (1941) Compt. rend. soc. Biol. 135, 875.

Gautheret, R. J. (1942a) Manuel technique des tissus vegetaux, Paris, Masson et Cie p. 172.

Gautheret, R. J. (1948) Compt. rend. soc. biol. 142, 808.

Gautheret, R. J. (1954) Annee. biol. 30, 202.

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

Gautheret, R. J. (1955a) Annee. biol. 31, 145.

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

Haberlandt, G. (1902) Sitzungsber, Akad. Wiss. Wien, Math-natur. Kl. 111, 69.

Hawk, P. B. and Bergeim, O. (1944) Pract. Physiol. Chem. XI ed. p.468.

Harrison, R. G. (1907) Proc. soc. Exp. Biol. Med. 4, 140.

Heller, R. (1949) Compt. rend. soc. biol. 143, 335.

Henderson, J. H. M., Durell, M. E. & Bonner, J. (1952) Amer. Jour. Bot. 39, 467.

Hildebrandt, A. C., Riker, A. J. & Duggar, B. M. (1946a) Amer. Jour. Bot. 33, 591.

Hildebrandt, A. C. & Riker, A. J. (1949) Amer.Jour.Bot. 36, 74.

Hildebrandt, A. C. & Riker, A. J. (1953) Amer.Jour.Bot. 40, 66.

Hildebrandt, A. C., Riker, A. J. and Duggar, B. M. (1955)
Amer. Jour. Bot. 32, 357.

Hofmann, K., Neals, D. B. and Panos, C. (1957)
Jour. Biol. Chem. 223, 349.

Hooke, R. (1667) Micrographia, 1665 ed. Lon. Pritzel No.4193.

Hotson, H. H. (1953) Phytopathol. 43, 360.

Jacquiot, C. (1951) C. R. Acad. Sci. (Paris) 233, 815.

Jacquiot, C. (1955) C. R. Acad. Sci. (Paris) 240, 557.

Jagendorf, A. T. (1952) Am. Jour. Bot. 39, 546.

Jagendorf, A. T. and Naylor, A. W. (1952) Bot. Gaz. 114, 334.

Johri, B. M. and Singh Bajaj, Y. P. (1963) Proc. Plant Tissue
& Organ Culture, Symposium, Delhi, p. 292.

Jones, L. E., Hildebrandt, A. C., Riker, A. J. and Wu, J. H. (1960)
Amer. Jour. Bot. 47, 460.

Kemmer, E. (1928) Arch. exp. Zellforsch. 7, 1.

Khalifah, R. A. and Lewis, L. N. (1963) Science 142, 399.

Kordan, H. A. (1958) Science 129, 779.

Kordan, H. A. (1963) Bull. Torr. Bot. Club, 90, 293.

Kotte, W. (1922) Beitr. allg. Bot. 2, 413.

Lamport, D. T. A. (1964) Exptl. Cell Research 33, 195.

Lavee, S. (1963) Science 142, 583.

Letham, D. S. (1958) Nature 182, 473.

Letham, D. S. (1960) Nature, 188, 425.

Letham, D. S. (1963) Colloq. Intern. Cent. Natl. Res. Sc.(Paris)
123, 109.

Loeffler, J. E. and Van Overbeek, J. (1963) Colloq. Intern. Cent.
Natl. Sc. (Paris) 123, 77.

Loewenberg, J. R. and Skoog, F. (1952) Physiol. Plant. 5, 33.

Maheshwari, P. and Kantha, K. (1965) Proc. Tissue Culture Seminar, Baroda, p. 542.

Main, E. (1963) Colloq. Intern. Centr. Natl. Sc. (Paris) 123, 103.

Mascarenhas, A. F., Sayagaver, B. M. and Jagannathan, V. (1965) Proc. Tissue Cult. Seminar, Baroda, p. 214.

Melchers, G. and Engelmann, L. (1955) Naturwissensch. 20, 564.

Miller, C. O., Skoog, F., Von Saltza, M. H. and Strong, F. M. (1955) Jour. Am. Chem. Soc. 77, 1392.

Mitra, J. and Maul, K. N. (1964) Ind. Jour. Expt. Biol. 2, 49.

Mitra, J., Mapes, M. O. and Steward, F. C. (1960) Am. Jour. Bot. 47, 357.

Mitra, J. and Steward, F. C. (1961) Am. Jour. Bot. 48, 358.

Mohan Ram, H. Y. and Steward, F. C. (1964) Canad. Jour. Bot. 42, 1559.

Mohan Ram, H. Y. and Wadhi, M. (1965) Proc. Tissue Culture Seminar, Baroda, p. 531.

Morel, G. (1946) C. R. Acad. Sci. (Paris) 223, 166.

Morel, G. and Wetmore, R. H. (1951) Am. Jour. Bot. 38, 141.

Muir, W. H., Hildebrandt, A. C. and Riker, A. J. (1954) Science, 119, 877.

Muir, W. H., Hildebrandt, A. C. and Riker, A. J. (1958) in Ann. Rev. Microbiol. 12, 469.

Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473.

Nickell, L. G. (1950) Am. Jour. Bot. 37, 829.

Nickell, L. G. and Burkholder, P. R. (1950) Am. Jour. Bot. 37, 538.

Nickell, L. G. (1952) Bull. Torr. Bot. Club, 79, 427.

Nickell, L. G. and Brakke, M. K. (1954) Am. Jour. Bot. 41, 390.

Nickell, L. G. (1956) Proc. Nat. Acad. Sci. Wash. 42, 848.

Nickell, L. G. and Tulecke, W. (1959a) Bot. Gaz. 120, 245.

Nickell, L. G. and Tulecke, W. (1960) J. Biochem. microbiol. Techn. Engng. 2, 207.

Nickell, L. G. (1962) Adv. in Appl. Microb. Academic Press, 4, 222.

Nitsch, J. P. (1951) Am. Jour. Bot. 38, 566.

Nitsch, J. P. and Nitsch, C. (1956a) Pl. Physiol. 31, 94.

Nitsch, J. P. (1959) Bull. Soc. Bot. Fr. 106, 420.

Nitsch, J. P. (1963) Proc. Plant Tissue & Organ Culture, Symp. Delhi, p. 198.

Nobecourt, P. (1937) Cr. Acad. Sci. Paris, 231, 521.

Nobecourt, P. (1939a) Cr. Soc. Biol. Paris, 130, 1270.

Overbeek, van J., Conklin, M. E., Blakeslee, A. F. (1941) Science 94, 350.

Paleg, L. G. (1962) Pl. Physiol. 37, 579.

Partridge, S. M. (1948) Biochem. Jour. 42, 238.

Pelet, F., Hildebrandt, A. C., Riker, A. J. and Skoog, F. (1960) Am. Jour. Bot. 47, 186.

Plaisted, P. H. (1958) Contr. Boyce Thom. Inst. 19, 231.

Pollard, J. K., Shantz, E. M. and Steward, F. C. (1959) Pl. Physiol. 34, Suppl. VIII.

Pollard, J. K., Shantz, E. M. and Steward, F. C. (1961) Pl. Physiol. 36, 492.

Porter, C. A., Margolis, D. and Sharp, P. (1957) Contr. Boyce Thom. Inst. 18, 467.

Powell, L. E. (1964) Nature 204, 602.

Raghavan, V. and Jacobs, W. P. (1961) Am. Jour. Bot. 48, 751.

Reinert, J. (1956) Science 123, 457.

Reinert, J. and White, P. R. (1956) Physiol. Plant. 9, 177.

Riker, A. J. and Gutsche, A. E. (1948) Am. Jour. Bot. 35, 227.

Riker, A. J. and Hildebrandt, A. C. (1958) Ann. Rev. Microbiol. 12, 469.

Risser, P. G. and White, P. R. (1964) Physiol. Plant. 17, 620.

Robbins, W. J. (1922a) Bot. Gaz. 73, 376.

Sachs, J. (1880) Arb. Bot. Inst. Wurzburg 2, 452.

Sachs, J. (1882) Arb. Bot. Inst. Wurzburg 2, 699.

Sandell, E. B. (1944) Color detm. of trace metals. Interscience Publishers, New York.

Sastri, R. L. N. (1963) Plant Tissue & Organ Culture, Symp. Delhi, p. 105.

Sayagaver, B. N. (1965) M.Sc. Thesis, Bombay University.

Schleiden, M. J. (1838) Arch. Anat. Physiol. u. wiss. Med. 1838, 137.

Schwann, Th. (1839) Microskopische Untersuch. uber die Ubereinstimmung in der Struktur und dem Wachstume der Tiere and Pflanzen, Leipzig, Nr. 176, 1910.

Shantz, E. M. and Steward, F. C. (1952) J. Am. Chem. Soc. 74, 6133.

Shantz, E. M. and Steward, F. C. (1955) J. Am. Chem. Soc. 77, 6351.

Shantz, E. M., Mears, K. and Steward, F. C. (1958) Plant Physiol. 33, Suppl. XVI.

Shantz, E. M. and Steward, F. C. (1963) Coll. Int. Centr. Natl. Res. Sci. (Paris) 123, 59.

Skoog, F. (1944) Am. Jour. Bot. 31, 19.

Skoog, F. and Miller, C. O. (1957) Symp. Soc. Exptl. Biol. 11, 118.

Spencke, T., McCloskey, P., Smith, W. K., Scot, T. A. and Hussey, H. (1964) Nature (Lond.) 201, 614.

Srivastava, B. I. H. (1963) Plant Physiol. 38, Suppl. XLIII.

Steeves, T. A. and Sussex, I. M. (1952) Nature, 170, 672.

Steinhart, C. E., Standifer, L. C. and Skoog, F. (1961) Am. Jour. Bot. 48, 466.

Steward, F. C. and Caplin, S. M. (1951) Science, 113, 518.

Steward, F. C. and Pollard, J. K. (1958) Nature, 182, 628.

Steward, F. C., Mapes, M. O. and Smith, J. (1958) Am. Jour. Bot. 45, 693.

Steward, F. C., Halma, A. C., Freiberg, S. R., Hegarty, M. P., Pollard, J. K., Rabson, R. and Barr, R. A. (1960) Ann. Bot. 24, 83.

Steward, F. C., Pollard, J. K., Mapes, M. O., and Mitra, J. (1961) 19th Ann. Growth Symp. New York, p. 193.

Steward, F. C. (1963) Proc. Plant Tissue & Organ Culture Symposium, Delhi, p. 1.

Strauss, J. and La Rue, C. D. (1954) Am. Jour. Bot. 41, 687.

Strauss, J. (1960) Am. Jour. Bot. 47, 641.

Street, H. E. and McGregor, S. M. (1952) Physiol. Plant. 5, 248.

Street, H. E. and Henshaw, G. G. (1963) Symp. Soc. for Exptl. Biol. No. XVII, 234.

Sussex, I. M. and Steeves, T. A. (1953) Nature, 172, 624.

Tamaoki, T., Hildebrandt, A. C., Burris, R. H., Riker, A. J. and Nagihara, B. (1960) Plant Physiol. 35, 942.

Tamaoki, T., Hildebrandt, A. C., Burris, R. H. and Riker, A. J. (1961) Plant Physiol. 36, 347.

Tepfer, S. S., Greyson, R. I., Craig, W. R. and Hindman, J. L. (1963) Am. J. Bot. 50, 1035.

Thimann, K. V. (1963) Ann. Rev. Plant Physiol. 14, 1.

Torek, de D., & Thimann, K. V. (1961) Physiol. Plant. 14, 543.

Torrey, J. G. (1957) Proc. Nat. Acad. Sci. U. S. 43, 887.

Torrey, J. G. and Shigemura, Y. (1957) Am. Jour. Bot. 44, 334.

Torrey, J. G. and Shigemura, Y. (1958) Science, 128, 1148.

Torrey, J. G. (1961) Exptl. Cell Research, 23, 281.

Torrey, J. G. and Reinert, J. (1961) Plant Physiol. 36, 433.

Torrey, J. G., Reinert, J. and Morkel, N. (1962) Am. Jour. Bot. 49, 420.

Tryon, K. (1955) Am. Jour. Bot. 42, 604.

Tulecke, W. (1953) Science, 117, 599.

Tulecke, W. (1959) Bull. Torrey Bot. Club, 86, 283.

Tulecke, W. and Nickell, L. G. (1959) Science, 130, 883.

Tulecke, W. (1960) Plant Physiol. 35, 19.

Tulecke, W. and Nickell, L. G. (1960) Trans. N.Y.Acad.Sci. 22, 196.

Tulecke, W., Weinstein, L. H., Rutner, A. and Laurencot, H.T. (Jr.) (1962) Contr.Boyce Thom.Inst. 21, 291.

Uspenski, E. E. and Uspenskaia, W. J. (1925) Ztschr.Bot. 17, 273.

Varner, J. E. (1962) Plant Physiol. 39, 413.

Vasil, I. K. (1963) Proc. Plant Tissue & Organ Culture, Symp. Delhi. p. 230.

Vasil, I. K., Hildebrandt, A. C. and Riker, A. J. (1964a) Am. Jour. Bot. 51, 677.

Ward, M. (1964) Nature, 204, 400.

Weinstein, L. H., Nickell, L. G., Laurencot, H. J. and Tulecke, W. (1959) Contrib. Boyce Thom. Inst. 20, 239.

Weinstein, L. H., Tulecke, W., Nickell, L. G. and Laurencot, H. J. (1962) Contrib. Boyce Thom. Inst. 21, 371.

West, F. R. and Mika, E. S. (1957) Bot. Gaz. 119, 50.

White, P. R. (1934a) Plant Physiol. 9, 585.

White, P. R. (1939a) Am. Jour. Bot. 26, 59.

White, P. R. (1942) Plant Physiol. 17, 153.

White, P. R. and Braun, A. C. (1942) Cancer Res. 2, 597.

White, P. R. (1943) A Handbook of Plant Tissue Culture, Lancaster Press.

White, P. R. (1953) Am. Jour. Bot. 40, 517.

White, P. R. (1954) The Cultivation of Animal and Plant Cells, Ronald Press Co.

White, P. R. (1959) The Cell, Vol. I, Ed. Brachet, Mirsky.

White, P. R. and Risser, P. G. (1964) Physiol. Plant, 17, 600.

Wiggans, S. C. (1954) Am. Jour. Bot. 41, 321.

Zwar, J. A. and Skoog, F. (1963) Austral. Jour. Biol. 16, 1293.