THE UNIVERSITY OF ASTON IN BIRMINGHAM

PHYTOHORMONES AND THE CULTURE OF *AGARICUS BISPORUS* (LANGE)PILAT.

by

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Atta A.Kadhim Hussain ,B.Sc.,M.Sc. Doctor of Philosophy 1981

SUMMARY

Addition of synthetic phytohormones (auxin,gibberellin and cytokinin)to Agaricus bisporus culture media promoted mycelial growth significantly at concentrations of 10^{-6} - 10^{-7} M. Most of the tested phytohormones were more effective in growth promotion in liquid culture rather than on semi-solid agar . Phytohormones stimulated hyphal cell division and cell elongation .

Increased yield was obtained by watering compost and casing soil with solutions of gibberellic acid ,α-naphthalene acetic acid and 2,4-dichlorophenoxy acetic acid ,while indole-3-acetic acid and kinetin were without marked effects .The average weight of fruit body was increased by addition of most of the tested phytohormones ,but it was at the expense of the number of sporophores . In general,synthetic phytohormones did not effect the shape of the sporophore,but stipe length and pileus diameter were increased by some phytohormones .

Malt extract was shown to have auxin,gibberellin and cytokinin activity which may explain its superiority in formation of primordia over other media . Addition of synthetic phytohormones to malt extract agar accelerated primordium initiation and significantly increased number over the control .

Compost and casing soil contained appreciable amounts of the three groups of phytohormones (auxins,gibberellins and cytokinins). The fluctuation in levels of phytohormones in compost and casing soil were related to fluctuations in the population of bacteria and fungi.

Phytohormones play a role in regulating the interaction between A.bisporus and other micro-organisms in artificial culture .

The mycelium of A.bisporus and selected isolates of bacteria and fungi synthesise and utilize auxin,gibberellin and cytokinin .

High concentrations of phytohormones in the primordium stage compared to those in mycelium and fruit body stages,and the inverse correlation between phytohormone content and the age of developing fruit bodies, suggest that phytohormones function in the development of fruit bodies. In general,phytohormones influence vegetative growth ,transition from vegetative to reproductive growth and the development of fruit bodies of Agaricus bisporus.

KEY WORDS

PHYTOHORMONES AGARICUS BISPORUS

DECLARATION

I declare that the work described in this thesis is the result of my own investigations, except where reference is made to published literature and where assistance is acknowledged, and that the work has not been submitted for any other award.

A.A.K. HUSSAIN

Candidate

April 1981

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INTRODUCTION

INTRODUCTION

Phytohormones are known to perform an important role in controlling growth and development in higher plants and following many studies on their mechanisms and functions, phytohormone action is now applied in many different ways in agriculture.

In contrast, the role of phytohormones in the growth and differentiation of fungi is not clear, despite many attempts to determine a functional role.

Phytohormones have been isolated from mycelium, culture filtrates and fruit bodies of various fungi including several members of the Agaricales. Also, the promoting effect of some phytohormones on mycelial growth of some fungal species is well known. Tropic responses to applied phytohormones have led to the conclusion that auxin, or indole acetic acid, the principal plant growth hormone is not involved in tropic responses in fungi.

A "Mushroom growth hormone", however, was postulated by Hagimoto and Konishi (1959) to explain stipe curvatures of the cultivated mushroom Agaricus bisporus which resulted after the removal of the lamellae. It was suggested that fruit body development of A.bisporus depends on a substance or substances (the mushroom growth

hormone) produced in the lamellae and is transferred to the growth zone in the stipe and the cap margin.

In higher plants, phytohormones play a vital role in the transition from vegetative to reproductive growth. In A.bisporus the transition from vegetative to reproductive growth is controlled by a complex of physical, chemical and biological factors. Especially relevant is the requirement for certain bacteria for this transition to occur. It is known, for example, that most bacteria are able to synthesise at least one phytohormone. Any links between the development of initials, primordia and fruit bodies and phytohormones have not been established.

The process of artificial mushroom culture is a mixed culture of micro-organisms involving especially, bacteria, fungi and actinomycetes and all of which may contribute to the success of substrate colonisation (selectivity) and productivity. By-products of their activities may be inhibitory or stimulatory to A.bisporus and these substances inevitably include substances which may be classed as phytohormones.

Also, since A.bisporus is grown on a cereal straw, this also contributes to the total phytohormone content

of substrate environment.

The purpose of this study is to establish the extent to which the principal phytohormones, auxins, gibberellins and cytokinins, play a role in the growth and development processes in the cultivated mushroom A.bisporus.

SECTION 1

REVIEW OF RELEVANT LITERATURE

REVIEW OF RELEVANT LITERATURE

1.1 The chemical nature and physiological action of phytohormones

The widely accepted definition of a plant hormone was given by Thimann (1948). It is,

> "an organic substance produced naturally in higher plants, controlling growth or other physiological functions, and active in minute amounts".

Thimann favoured the term "phytohormone" for these substances to distinguish them from the animal hormones.

In recent years, it has become clear that control of growth and development in plants is shared by several hormones, especially those grouped as auxins, cytokinins, and gibberellins. These interact to induce a wide range of responses and they also interact with other naturally occurring inhibitors, such as phenols, flavonols, and abscisic acid.

1.1.1 The auxins

Pincus and Thimann (1948) defined auxin as,

"an organic substance which promotes growth (irreversible increase in volume) along the longitudinal axis, when applied in low concentrations to shoots of plants freed as far as practicable from their own inherent growth promoting substances."

Indole-3-acetic acid (IAA) was first isolated as an auxin from human urine and yeast plasmolysate by Kogl, et. al. (1934). Subsequently, it has been isolated from culture filtrates of micro-organisms in particular from fungi. Nielsen, et. al. (1930), Nielsen (1928), Bonner (1932), Borzini (1935), Dolk and Thimann (1932), Thiman, et. al. (1933) and Thimann (1935) isolated and identified indole-3-acetic acid (IAA) from Rhizopus suinus culture filtrates. Levan, et. al. (1943) and Gruen (1959) isolated IAA from Phycomyces blakesleeanus culture medium. Hirata (1958) showed that a number of Fusaria synthesized both auxin and gibberellin.

Many workers have isolated at least one auxin

from many species of Basidiomycetes. Nielson (1930), Urayama (1956), and Almoslechner (1934) isolated an auxin from the sporophore of Agaricus compestris and Boletus edulis.

Although it was once thought that IAA was a product only of micro-organisms more recently IAA has been isolated from most species of higher plants (Audus ,1972 a).

A close chemical relative of IAA is the amino acid tryptophane which occurs in all living plant cells, usually as a constituent part of the proteins in the protoplasm. Several workers have shown that various micro-organisms have the ability to convert tryptophane to IAA (Thimann , 1935 ; Konishi, et. al. , 1961). Also Wildman, et. al. (1947) and Gordon (1954, 1961) demonstrated the ability of plants to convert tryptophane to IAA.

Audus (1972 a) illustrated the probable biochemical sequence whereby IAA could be formed from tryptophane, as follows:

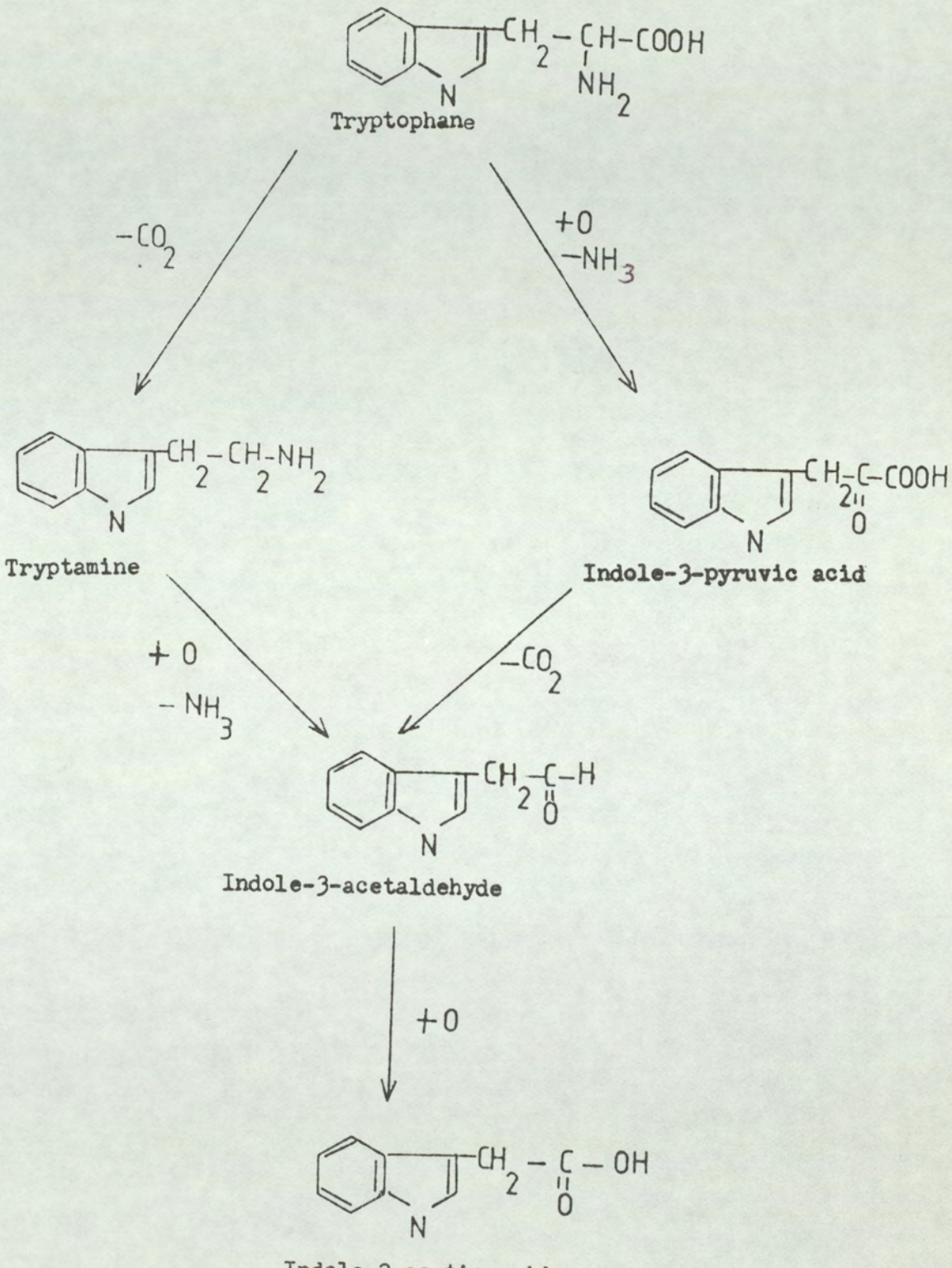


Diagram showing the probable pathways of the biosynthesis of indole-3-acetic acid (IAA).

Leopold (1955 a) suggested the basic molecular requirements for auxin activity. These are as follows:

(a) A ring-system as a nucleus.

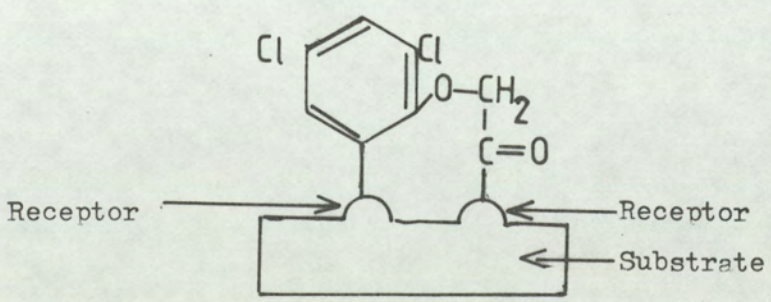
(b) At least one double bond in this ring.

(c) A side chain possessing a carboxyl group.

(d) At least one carbon atom between the ring-system and the carboxyl group in the chain.

(e) A particular spatial relationship between the ring-system and the carboxyl group.

He suggested that auxins act by becoming attached to other substrates at two points. The ring can be attached to some receptor (at the ortho-position in the case of phenoxy acetic acid), and the acidic group can combine with the same receptor to give two points of attachment as follows:



The most common action of auxin is to promote cell enlargement (Thimann, 1969). Auxin may cause cell division in some tissues but not all. The most apparent action of auxin in controlling cell division is in the cambium (Snow, 1935; and Soding, 1936). It has since been widely observed. Patau, et. al. (1957) found that cell division begins as soon as IAA is added to tobacco pith culture, but cytokinin is needed for continuous growth.

Auxin delays the abscission process for many days if synthetic IAA is applied. La Rue (1935), obtained comparable results, with both Coleus and Ricinus, by applying pure IAA.

Although, it has been shown that auxin production is widespread among fungi belonging to different taxonomic groups, there is no clear-cut evidence for a growth-regulating role of auxins, particularly IAA, in fungi.

Auxins induce some abnormalities and morphological changes, usually at high concentrations. Richards (1949), found that auxin induced change in colony morphology of Phycomyces, Aspergillus, Neurospora and Schizophyllum. Hsia, et. al. (1951) showed that the application of an auxin caused increase sectoring in colonies of Helminthosporium.

Synthesis of some fungal metabolites are increased

by addition of auxins. Grace (1937) found that fermentation by Saccharomyces was strongly stimulated by addition of IAA and ∝-naphthalene acetic acid (NAA). wai (195) showed that IAA induced the production of citric acid by Aspergillus niger.

Auxin exerts two opposing kinds of action, inhibition and promotion or acceleration. Biggs and Leopold (1958) suggested that the action of auxin simply followed a classical optimum concentration curve, low concentrations promoting and high concentrations inhibiting.

1.1.2 Gibberellins

Gibberellins are a second group of phytohormones in higher plants. One of the most pronounced effects is to increase the growth rate of dwarf stems. For example, the addition of gibberellin to a cabbage plant converts the head or dwarf stem into a stem which is six-eight feet tall.

Cleland (1969) defined a gibberellin as,

> "A compound which is active in gibberellin bioassays and possesses a gibbane ring skeleton."

Gibberellin-like substances were defined by Phinney and West (1960) as,

> "Substances not containing the gibbane

ring which nevertheless have biological activity in gibberellin bioassay systems"

Gibberellins are widely distributed in nature and have been detected in most species of Angiosperms, in some species of Gym nosperms, in ferns, algae, fungi and bacteria. Also, all the organs of higher plants contain gibberellins, but the highest levels have been found in seeds.

The first isolation of gibberellin was done by Japanese workers from culture fitrates of Gibberella fujikuroi and Curtis, et. al. (1954) also isolated a compound with similar properties from Gibberella fujikuroi and they called this substance gibberellin.

In order to understand the role of gibberellin in the plant the mechanisms by which gibberellin exerts its effects should be known. Some investigators suggested that gibberellin exerts its physiological effects by altering the auxin status of the tissue and morphogentic effects of gibberellin are due to an increase in the endogenous auxin level. Brain and Himming (1958), Sastry and Muir (1963), showed that the level of diffusible auxin that could be obtained from unfertilised tomato ovaries and from species of dwarf pea stems was markedly increased by gibberellin application. However in some cases gibberellin does not act by increasing the endogenous auxin level. In such cases gibberellin acts

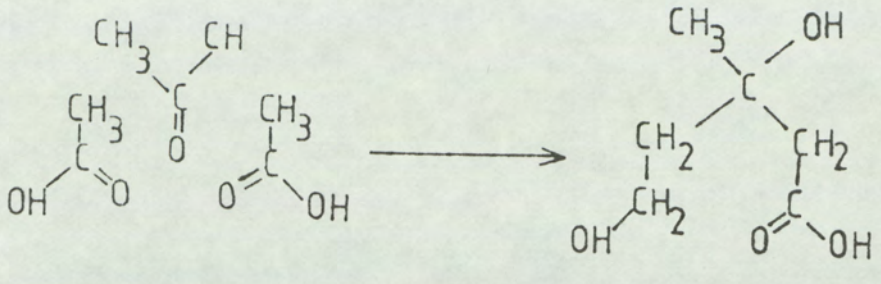
with auxin rather than through auxin to exert its response.

A possible mode of action, which has received increasing attention, is that it operates at the gene level to cause de-repression of specific genes. The activated gene being responsible for observed morphogentic changes through the production of new enzymes. The most extensive evidence to support such a mode of action emanates from the studies of Varner et. al. (1965) on the induction of α-amylase in the aleurone layer of the barley endosperm. In the absence of gibberellin, the aleurone cells endosperm contain only a trace amount of α-amylase after eight hours.

Birch et. al. (1958) concluded that the starting point for biosynthesis of gibberellin is acetic acid and the intermediate compound is mevalonic acid. They used acetate and mevalonic acid containing radioactive C^{14} in the culture of Gibberella. Subsequently, they obtained radioactive gibberellins.

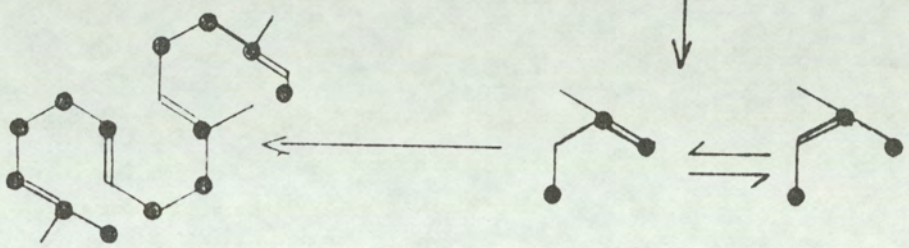
Cross et. al. (1964) studied in more detail the metabolites of Gibberella using isotopes searching for the main precursor of gibberellin. They found that (-)-kaurene is the main precursor of the gibberellin. Subsequently many workers supported Cross' hypothesis, notably, Upper and West (1967), Shechter and West (1969), Cavel and MacMillan (1967), and Hanson and White (1969).

Audus (1972b) illustrated the probable pathways of the biosynthesis of gibberellins, as follows:



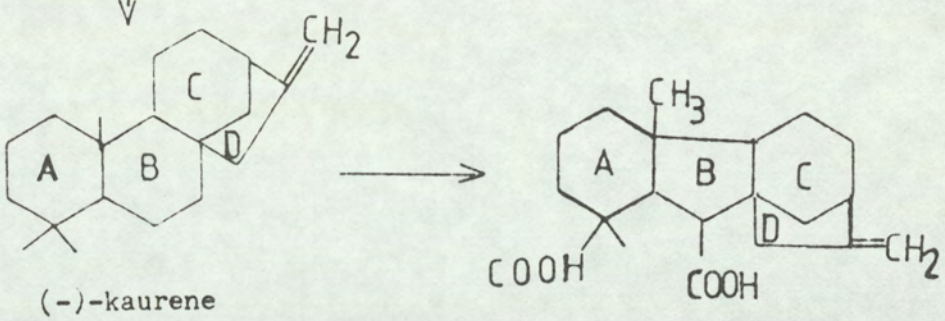
Three acetic acids

Mevalonic acid



Geranyl -Geraniol

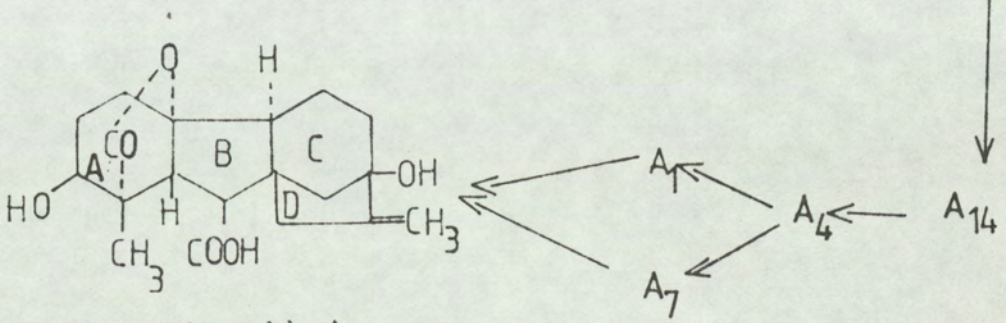
Isopentenyl-and dimethylallyl units



(-)-kaurene

(Tetracyclic ditepenoid)

Gibberellin A_{12}

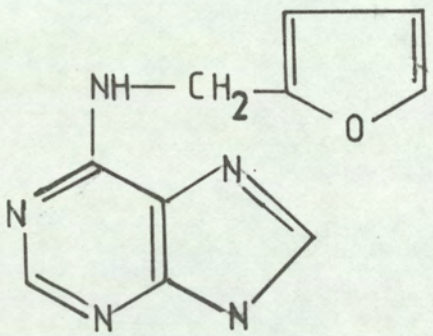


Gibberellic acid A_3

Diagram showing the probable pathways of the biosynthesis of the gibberellins .

1.1.3 Cytokinins

The cytokinins are chemicals which promote cytokinesis in cells of various plant origin (Skoog, Strong and Miller 1965). Because of their specific effect on cytokinesis the first cytokinin was named "kinetin". Kinetin was found to contain only carbon, hydrogen, nitrogen and oxygen in the ratio C_{10}, H_9, N_5, O and to absorb strongly in the ultraviolet with a single band near 268 mμ (Miller et. al. 1955).



Structure of Kinetin

The first isolation of cytokinin was done by Jablonski and Skoog (1954) from tobacco stems. Coconut milk and malt extract were also shown to contain high cytokinin activity. Miller et. al. (1955) demonstrated

high cytokinin activity from yeast extract and autoclaved DNA. They also showed (Miller et. al. 1956) that cell division in tobacco pith was induced by the application of cytokinin at concentrations of only 1.Oppm when auxin was also present in the medium.

Miller (1961), pointed out that at least one non-purine with cytokinin activity, N, N-diphenylurea may be used by the cell as a precursor of a kinetin-type compound.

Kinetin was originally derived from the degradation of DNA by slow spontaneous process or by autoclaving fresh DNA in weakly acid aqueous slurries. Hall et. al. (1955) synthesized kinetin from deoxydenosine. Hamzi et. al. (1964), Leonard et. al. (1966), Skoog et. al. (1967) and Skoog et. al. (1969) found that kinetin was produced by conversion of 1-, 3- or 9- substituted addenine derivatives to their respective N^6-isomers and then they concluded that trace amounts of kinetin might be expected in all cells, and perhaps in physiological significant concentrations at least in wounded tissues.

The property most characteristically associated with cytokinins is their stimulation of cell division in plant tissue culture. Das et. al. (1956) demon-

strated that with IAA by itself some mitoses occurred in the tissue resulting primarily in binucleate cells but only a few of the mitoses were followed by cell division. Kinetin in combination with IAA, induced many mitosis virtually all of which were followed by cytokinesis.

Cytokinins are reported to increase the rate of cell division in several micro-organisms, notably, a protozoa Paramecium caudatum (Guttman and Back, 1960) and bacteria such as Escherichia coli (Kennell, 1959). Also, the growth of several organisms including a thermophilic bacterium Clostridium thermocellum, has been stimulated by applying cytokinins(Maruzzella et. al. , 1963 ; Quinn et. al. , 1963) . The fertility of a fungus, a strain of Neurospora crass, was increased by kinetin(Lee, 1961).

Cytokinins stimulate protein synthesis in higher plants. Osborne (1962) demonstrated kinetin stimulated an increase in protein synthesis in leaf discs and he concluded that a primary action of kinetin might be to regulate RNA synthesis with a resulting regulation of one of the steps leading to protein synthesis.

Richmond and Lang (1957) found that kinetin delay-

ed the senescence of detached Cocklebur leaves. It has since been found that the senescence of detached leaves of many species, is delayed by other cytokinins as well.

Several workers have found that cytokinin enhances the synthesis of nucleic acid in many species of higher plants, notably, Guttman (1957) who reported an increase in the amount of RNA in onion roots treated with kinetin. Similar results have been reported by many investigators including Olszewska (1959), Wollgiehn (1965), Osborne (1962), and Jensen et. al. (1964).

1.2 Phytohormones and fungi

1.2.1 Tropisms of fungi and its mechanism

Numerous investigators have studied the geotropic and phototropic responses of fungi but there is no real evidence to demonstrate that tropisms in fungi involve an auxin mechanism similar to that of higher plants. Hawker (1950) obtained curvatures in the stipe of Collybia velutipes by removing half of the pileus and also by placing a block of agar containing fruit body extract asymmetrically on the decapitated stipe. Branbury (1952) found that the unilateral applications of IAA had no effect on Phycomyces sporangiophores.

Jefferys et. al. (1956) studied the mechanism of

tropisms in Coprinus sterquilinus, to determine whether the geotropic and phototropic responses of this fungus involve an auxin mechanism similar to that of higher plants. They found no significant curvatures by removing the pileus and placing unilaterally on the stump agar blocks containing different concentrations of IAA, NAA and diffusate of Corpinus sterquilinus. Subsequently, they concluded that the system governing tropism in Coprinus sterquilinus differs from that of higher plants. From studies with Phycomyces sporangiophores Thimann and Gruen (1960) came to the same conclusion.

Hawker (1950) suggested that geotropic curvature in fungal fruit bodies is due to an unequal distribution of some growth regulator leading to growth acceleration on one side. This was supported by Hagimoto (1963) who found the extract from the gills of Agaricus bisporus, caused curvatures in the stipe of Agaricus bisporus itself. He called the active substance a "mushroom growth hormone" and he attributed the curvature in the stipe to an unequal distribution of this substance.

Studying the interrelationship between cap and stipe might be used to reveal the endogenous growth regulation in Basidiomycetes. Gruen (1963) tried to use this concept in order to determine the mechanism involved in fungal tropisms, using the techniques of decap-

itation and partial removal of the cap of Agaricus bisporus. He found that, when the cap or the lamellae alone were left at one side of the stipe during the early stage of elongation, strong negative stipe curvatures resulted, while such curvatures were small or absent when the lamellae were removed from the cap slice. He suggested that the lamellae have specific action in the synthesis of growth factors. Gruen in his suggestion was very close to the hypothesis which explains the curvature in higher plants, as an unequal distribution of auxins throughout the coleoptile.

1.2.2 Phytohormones and the growth of fungi

Fraser (1953) found that IAA at concentrations of 5-100 ppm, markedly increased the growth of Psalliota hortensis (Agaricus bisporus) in liquid cultures, particularly in the presence of thiamin and biotin. Fraser suggested that IAA may play a part in the growth processes of this fungus similar to that in higher plants.

Richards (1949) studied with more detail the effect of IAA and several synthetic auxins on the growth of four fungi, Phycomyces blakesleeanus, Aspergillus candidus, Neurospora tetrasperma, and Schizophyllum commune. At concentrations of 0.001 ppm and 0.0001 ppm of IAA, an increase in the growth rate was obtained with the ex-

ception of Schizophyllum commune.

Aleksandrove (1964) claimed that an increase in Agaricus compestris mycelial growth and basidiocarp production was obtained by adding gibberellic acid to compost in artificial cultures. Pegg (1973) reported a reduction in fresh weight and dry weight of fruit body of Agaricus bisporus when gibberellins were applied to the pilei of young expanding sporophores as aqueous solutions. Negrutskii (1963) found that gibberellins retarded growth of Famitopsis annosa and Santoro and Casida (1962) showed the growth of Amanita caesaria, Amanita muscaria, Amanita rubescens, Boletus bicolor and Boletus lutens were retarded by addition of gibberellins to culture media.

In some instances phytohormones inhibit the growth of fungi at high concentration, while they have no pronounced effect at very low concentrations. For example, Aschan and Aberg (1958) who noted that high concentrations of IAA proved toxic whereas low concentrations had no effect on the growth of Collybia velutipes. Bhargava (1946) found that the addition of β-indole-3-acetic acid at low concentrations had no effect on some members of the family Saprolegniaceae, while higher concentrations retarded their growth. The same results were obtained by Murdia (1939).

There is very little information in the literature on the effect of cytokinin except for a report by Lee (1961) that the fertility of some strains of Neurospora crassa was increased by kinetin.

1.2.3 Biosynthesis of phytohormones by fungi

It has been known for a long time that most fungi synthesize IAA from its main precursor "tryptophane" and other organic substances. Thimann and Dolk (1933) isolated IAA from the culture of Rhizopus suinus, and studied with more detail the conditions governing auxin production, but they did not find any link between the growth of this fungus and auxin production. They regarded the auxin produced by the fungus as a by-product of its metabolism.

Wolf (1956) reported that cultures of the cedar apple rust fungus Gymnosporangium juniperi-virginianae Schw., produce IAA as a metabolic product. He also reported that the increase in auxin level in rust-infected plants was due entirely to the metabolic activities of the infecting rust fungus, the host merely serving as a source of the precursor, tryptophane.

Konishi and Hagimoto (1961) isolated IAA from the fruit body of Agaricus bisporus and they found that the

juice squeezed from the fruit body has an ability to convert L-tryptophane into IAA.

It appears that gibberellins and cytokinins are less widely distributed than IAA among fungi. Gibberellins produced by Gibberella fujikuroi, were found to produce in rice an elongation of the shoots, a characteristic of the Bakanae disease. Thus, several workers isolated these substances from the culture filtrates of Gibberella fujikuroi, including, Curtis and Cross (1954), and Borrow et. al. (1955). Pegg (1973) isolated gibberellin-like substances from Agaricus bisporus fruit body, and he concluded that gibberellins have a role in the growth and development of Agaricus bisporus. Cytokinins have been isolated from a few fungi, namely, Rhizopogon roseolus (Miller 1967), and two cultivated edible mushroom, Agaricus bisporus and Pleurotus sajor-caju (Dua and Jandaik 1979).

1.3 Growth and development of Agaricus bisporus

Under normal circumstances, Agaricus bisporus remains in the vegetative stage of growth in pure culture using the standard laboratory media. In the artificial methods adopted in commercial practice, the substrates and culture conditions are such that Agaricus bisporus grows in association with other micro-organisms (Hayes

1978 a and b).

The primary and nutrient rich substrate is prepared by composting wheat straw and manure mixtures according to set procedures which encourage an active thermophilic microflora involving three groups: bacteria, fungi and actinomycetes (Hayes 1968). The compost which results from this process is pasteurised usually by injecting live steam, before inoculating with a pure culture of Agaricus bisporus and incubating at 25°C for about fourteen days.

In order to induce the reproductive stage, it is necessary to "case" the colonised compost with a layer of soil normally a mixture of peat and calcium carbonate. Following the application of this layer cultures are incubated for a further seven to ten days at 25°C before the temperatures are lowered to 16-18°C and ventilated to maintain fresh air conditions and avoiding high carbon-dioxide concentrations.

Vegetative growth of Agaricus bisporus is established in the casing layer during the initial incubation at 25°C, but soon after lowering the temperature and ventilating, mycelial aggregates form into what are termed "primordia initials". A high proportion of these develop into "primordia" a proportion of which

develop into the characteristic Agaricus bisporus fruit bodies.

Under normal circumstances fruit bodies form approximately 21 days following the application of the casing soil and are harvested at the appropriate stage of maturity. Further "crops" of fruit bodies form at approximately weekly intervals, however, as "flushes" or "breaks". Normally the number of fruit bodies decline as cropping time progresses and after five or six "flushes", the cultures are disposed and new cultures established to maintain a continuous sequence of production.

The main purpose of artificial culture is to produce fruit bodies and therefore much attention has been given in recent years to the function and role of the casing layer by researchers. Research by Urayama (1961) and Eger (1961) demonstrated that sterilised casing soils did not support fruit body formation and studies by Hayes et. al. (1969) showed that Pseudomonas putida and Pseudomonas group IV isolates were associated with primordia and fruit body formation in casing soils.

1.3.1 Theories on the function of casing soil in the formation of primordia.

A number of different theories have been advanced on

the function of the casing soil inducing the important switch from vegetative to reproductive growth in Agaricus bisporus. Work by Lambert (1933), Tschierpe and Sinden (1964), Long and Jacobs (1968) stressed the importance of the carbon-dioxide levels in the control of primordium formation; their formation only taking place in atmospheres containing between 0.03% and 0.1% CO_2. Tschierpe (1959) postulated that the function of the casing was to produce a CO_2 gradient from air in the compost to the air above and Long et. al. (1968) suggested that fructification took place at a certain point in the region of CO_2 partial pressure gradient.

Mader (1943) postulated the existence of a substance acting as a stimulant to fructification when present in small amounts, but in larger amounts arresting sporophore growth and preventing fructification. Later, Stoller (1952) concluded that the function of casing was to provide an "alkaline oxygenated medium" for the destruction of an inhibitory volatile. Schisler (1957) proposed the existence of a high molecular weight hormone-like substance of low volatility which was produced by the mycelium.

A range of volatile compounds have been detected from growing mycelium, e.g., Lockard and Kneebone (1962),

Tschierpe and Sinden (1965), Staueble and Rast (1971), Turner, Wright, Ward, Osborne and Self (1975). However, the fact that casing soil when placed adjacent to compost rather than overlaying compost stimulates primordium formation suggests that the accumulation of volatile or indeed a gradient of gaseous products are not directly implicated. This is clearly shown by the "Halbschalentest" of Eger (1962) and Thomas *et. al.* (1964).

Following the work of Urayama (1961), Eger (1961) Thomas *et. al.* (1964) , Hayes *et. al.* (1969) concluded that the function of the casing soil was to provide a substrate in which stimulatory bacteria could successfully colonise together with the mushroom. Ethanol was shown by Hayes and Nair (1976) to be the most active compound of the known metabolites of *Agaricus bisporus* in stimulating bacteria.

A wide range of substances have been used to stimulate primordia in semi-solid media. These include haem compounds and chelating agents (Hayes 1972), biotin and phytohormones (Park *et. al.* 1969; Hayes, unpublished results).

Giovannozi-Sermanni *et. al.* (1974) studied the effect of *Arthobacter* species on sporophore formation

in terms of production of phytohormones. They found that this bacteria produces three groups of phytohormones (auxins, gibberellins and cytokinins) which, in turn, effected the sporophore formation in Agaricus bisporus. Also, an increase in yield was reported to have occurred by spraying bacteria extract on the casing soil.

Some workers attempted to clarify the relationship between the presence of the micro-organism and its influence on primordia initiation. Urayama (1967) concluded that the effect of Bacillus species on primordium initiation in Psilocybe panaeoliformis is related to its metabolites. He found that the fruit body promoting metabolites is neither an amino acid nor a phytohormone.

The influence of extracts obtained from different stages of fruit bodies of some Hymenomycetes on primordium initiation in Marasmius species has been studied by Urayama (1972). He found that the crude extract from young fruit body of Agaricus bisporus, Lentinus edodes, Flammulina velutipes and Pleurotus ostreatus induced primordium initiation in Marasmius species while the crude extract from mature fruit body of Agaricus bisporus or Lentinus edodes had almost nega-

ative effect on the primordium formation. He also purified the extract, then found the active substance in primordium initiation was free from volatile fatty acid, organic acid and phosphates.

1.4 Conclusions

A survey of the literature has revealed scattered and contradictory observations concerning phytohormones in the growth and development of Agaricus bisporus. Since phytohormones are a constituent of plants and since the substrates of Agaricus bisporus are of plant origin, it is clear that they form a part of the chemical and nutritional environment. Some workers have also succeeded in extracting some phytohormones from Agaricus bisporus mycelium and fruit bodies, but there is no evidence to suggest a possible role these substances may have in its growth and development. A more comprehensive and detailed study using modern techniques should establish their involvement in growth and development processes.

A wide range of investigations have been done which offer many attractive viewpoints on factors which govern the formation of primordia and fruit bodies of Agaricus bisporus in the casing layer. The inhibitory effects of carbon-dioxide on primordium and fruit body

formations are well known, but there is substantial evidence to suggest that a substance or substances may be produced in the casing layer which may act as a stimulator or an inhibitor or both according to its concentration. A "hormone-like substance" has been suggested by some workers.

The requirement for certain bacteria in the casing layer for primordium and fruit body development is well known. The fact that the entire process of mushroom culture is dependent on the activities of a mixed population suggests that these organisms by their activities may also influence the production and utilisation of phytohormones.

SECTION 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Isolation and maintenance of Agaricus bisporus

A commercially cultivated white strain of Agaricus bisporus (Lange) Pilat, was used throughout this investigation. The strain was isolated from grain spawn by transferring several grains aspetically onto a synthetic agar medium with a sterile forceps, and incubated at 25°C for 20-25 days. Cultures were maintained by subculturing on the same medium (section 2.2.2) at 25-day intervals.

2.2 Culture on synthetic agar

2.2.1 Inoculum

A. bisporus inoculum was prepared by aseptically removing agar blocks from the mid-point between the centre and edge of 25-day colony, using a number 3 cork borer before transferring to the synthetic agar medium in 9cm Petri plates.

2.2.2 Synthetic agar culture methods

A medium developed by Hayes (1972) was used with a slight modification indicated below. Its ingred-

ients were:-

Maltose	7.8 g	$CaCl_2$	0.2 g
Glucose	2.9 g	$FeSO_4$	0.001 g
Sucrose	0.27g	Na_2HPO_4	0.06 g
Dextrin	2.25g	Biotin	2.0 µg
*L-asparagine	2.0 g	Thiamin	8.0 µg
KCl	0.025g	Ethyl acetate	0.01 ml
$MgSO_4.7H_2O$	0.2 g	Oxiod agar	15 g
Deionized water	1000 ml		

Prior to autoclaving pH was adjusted to 7.6 by addition of N/10 NaOH.

The medium was autoclaved at 15 psi for 15 minutes but glucose, sucrose and L-asparagine were autoclaved separately and then added to the autoclaved medium at concentrations indicated above. Biotin, thiamin and ethyl acetate were filter-sterilised using "Millipore" filters, and added to the autoclaved medium immediately before gelling. Petri plates containing the agar medium were then inoculated with Agaricus bisporus and incubated at 25°C for four weeks.

2.2.3 Sterilisation of phytohormones

A stock solution of the tested phytohormones were sterilised by "Millipore" filters and pipetted into 100 ml of warm synthetic medium and thoroughly mixed before

* L-asparagine replaced casein hydrolysate which was used in original medium (Hayes, 1972) at a concentration of 1g/l.

pouring (20 ml) into 9 cm plastic Petri plates.

2.2.4 Growth measurement on agar

The growth of each colony was measured along three diameters and means calculated. The mean of five replicate colonies was calculated for each estimation of growth.

2.3 Culture in liquid medium-growth studies

A synthetic medium prepared as in section 2.2.2, was dispensed in 50 ml quantities into 150 ml Erlenmeyer flasks, sterilised by autoclaving at 15 psi for 15 minutes. After cooling the filter-sterilised vitamins and autoclaved glucose, sucrose and L-asperagine were added. Filter-sterilised hormones were pipetted into flasks at certain concentrations, while the same amount of sterilised deionised water was added to control flasks.

Culture flasks were aseptically inoculated by transferring a single disc of inoculum (section 2.2.1) to each flask and allowed to float on the surface before incubation for four weeks at 25°C.

Cultures were harvested by filtering off the mycelium on dried filter papers (Whatmann No. 1) of known weight. Finally the filter papers and mycelium

were dried at 100°C for 24 hours then cooled in a desiccator and weighed. Five replicates of each treatment were normally prepared, unless stated otherwise in section 3.

2.4 Solid substrate culture of Agaricus bisporus

2.4.1 Preparation of Agaricus bisporus spawn

Grains were soaked in water overnight, then dispensed in 500 ml Erlenmeyer flasks to which a small amount of chalk was added to adjust pH to 7-8. Flasks were then autoclaved at 15 psi for one hour daily for three days. After cooling they were inoculated with Agaricus bisporus inoculum and incubated at 25°C for 14-16 days. All flasks were then stored in a refrigerator when not in use.

2.4.2 Inoculation of compost

A pasteurised horse manure-straw compost obtained from W. Darlington & Sons Ltd., Rustington, Sussex, was inoculated by thoroughly mixing with grain spawn (1%) and then covered by polyethylene sheeting until the mycelium penetrated most of the compost. The compost was then mixed and compacted in 600 g quantities in autoclaved polypropyline growing pots (12 cm x 12 cm x 12 cm). Subsequently, they were incubated in a grow-

ing cabinet at 25°C and 80-90% RH (relative humidity) for 7-10 days when the mycelium covered the surface of the compost fully.

2.4.3 Casing soil

After the compost was fully colonised, all pots were cased with a 2.5 cm layer of commercial peat/ chalk casing soil.

Casing soil was prepared by thoroughly mixing Irish peat and "Morden 'R' chalk in the ratio of 2:1 (w/w). The mixture was watered before casing. Pots were incubated for a further 10 days at 25°C in the same cabinet and then at 17°C and RH 70-80% under constant ventilation.

2.4.4 Application of test phytohormone solutions

Known concentrations of phytohormones in 60 ml of water were uniformly sprayed on the surface of compost and/or casing soil at the following intervals: 5 days before casing, 5 days after casing, after first and second flush. Deionized water (60 ml) was added to untreated pots at the same intervals.

A phytohormone solution was prepared by dissolving a given amount of the phytohormone in a small amount of ethanol 90% (10:1, w/v) and diluting with deionized

water to make up to the volume required.

2.4.5 Harvesting and drying the sporophore

Sporophores were harvested at the open-cap stage of development by firstly twisting and then pulling. The bottom of the stipe, with soil adhering was cut and then fresh sporophores were weighed. Cap diameter and stipe length were measured after dividing the sporophore longitudinally.

Sporophores with 30-35 mm stipe length and 35-40 mm cap diameter were dried in an oven at 100°C for 48 hours cooled in a desiccator and then weighed.

2.5 Submerged culture of isolated bacteria

2.5.1 Techniques used in isolation of bacteria from compost and casing soil

Bacteria were isolated from compost and casing soil according to Hayes (1969). A peptone solution (1%) was used for primary isolation while nutrient agar was used for growing and subculturing isolated bacteria. Thus, several bottles containing 100 ml and 99 ml of peptone solution, as well as 99 ml of nutrient agar, were autoclaved and prepared in advance of sampling.

Samples (1 g) from compost were shaken with 100 ml

of peptone solution for 5-10 minutes. This was repeated for casing soil. This was the 10^{-2} dilution. One ml of the 10^{-2} dilution was pipetted into 99 ml peptone solution to make 10^{-4} diltuion. Similarly, the 10^{-6} and 10^{-8} dilution were prepared.

For nutrient agar plates, one ml of the 10^{-2} dilution was pipetted into 99 ml of nutrient agar to make the 10^{-4} dilution. Similarly, the 10^{-6}, 10^{-8}, and 10^{-10} dilution were prepared. The media were then dispensed into five 9 cm Petri plates. Subsequently, all plates were incubated at 25°C for 2-3 days and then the colonies of bacteria were counted.

2.5.2 Bacterium inoculum

The dominant bacterium at 10 days after casing was selected for study. An inoculum of this isolate was prepared by transferring a loopful to 150 ml Erlenmeyer flask containing 50 ml of a synthetic medium (section 2.2.2). The flask was incubated at 25°C on a rotary shaker for 3 days.

2.5.3 Submerged culture of bacteria for phytohormone tests

Submerged cultures of the isolated bacterium were prepared by dispensing a synthetic medium (section 2.2.2)

into 50 ml amounts in 150 ml Erlenmeyer flasks which were then autoclaved at 15 psi for 15 minutes. The flasks, after cooling, were inoculated by pipetting 1 ml of the inoculum (section 2.5.2) into each of three replicate flasks. Three control flasks were left without inoculation and served as control. All flasks were incubated at 25-27^{o}C on a rotary shaker for 7 days.

These cultures were used for phytohormone production and utilisation tests, but in the case of the latter a sterilised phytohormone was added to each flask, after autoclaving, at concentrations of 10 ppm. Three flasks were left without inoculation, served as control.

After 7 days of incubation, the culture flasks were centrifuged at 5000-6000 g for 30-40 minutes. The supernatants were individually taken and adjusted to appropriate pH (2.5 for IAA and GA analysis; 8.2 for cytokinin analysis).

2.6 Submerged culture of isolated fungus

2.6.1 Isolation technique

The technique used was based on that used by Hayes (1969) using 1% peptone solution and dextrose peptone

agar medium supplemented with rose bengal and aureomycin. Thus several bottles containing 100 ml and 99 ml of peptone solution, as well as 99 ml of dextrose peptone agar medium (peptone, 20 g; dextrose, 10 g; sodium chloride, 5 g; deionized water, 1000 ml) were autoclaved and prepared in advance of isolation.

Samples from compost and casing soil, each of 1 g fresh weight, were taken from materials (compost & casing) prepared for hormone analysis and suspended in 100 ml of peptone solution. They were shaken for 5-10 minutes before preparing serial dilutions.

Fungi were isolated on a dextrose peptone agar medium containing rose bengal(0.03 g/l) and aureomycin (0.002 g/l) which were added prior to setting. One ml aliquots of the diluted suspensions were pipetted into each of four bottles containing 99 ml of dextrose peptone agar medium, and shaken well before dispensing into Petri plates (9 cm). Thus four dilutions were prepared (10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10}), and each one was dispensed into five Petri plates. All the plates were incubated at 25°C for 5-7 days. The number of fungal colonies in each dilution was then calculated.

2.6.2 Fungal inoculum

The dominant species (Penicillium sps) was transferred to dextrose peptone agar and incubated at 25°C for 5-7 days for use as an inoculum in a liquid medium.

2.6.3 Submerged culture of Penicillium sps.

The isolated fungus was cultivated on Czapek Dox liquid medium (sodium nitrate, 2 g; potassium chloride, 0.5 g; magnesium glycerophosphate, 0.5 g; ferrous sulphate, 0.35 g; sucrose, 30.0 g; deionized water, 1000 ml; pH, 6.8) in submerged cultures. The medium was dispensed in 50 ml amounts into 150 ml Erlenmeyer flasks and autoclaved at 15 psi for 15 minutes. After cooling, the flasks were inoculated by transferring a single inoculum (section 2.6.2) and incubated at 25°C on a rotary shaker for 7 days, then the mycelium was harvested according to procedure presented in section 2.3, and dried. Each culture flask was filtered on Whatmann No. 1 filter paper, and the filtrate was adjusted to pH 2.5 for IAA and GA and 8.2 for cytokinin analysis.

2.7 Analytical Techniques

2.7.1 Nitrogen and protein estimation

Samples (60-80 mg) from dried and homogenized fruit

bodies were transferred with a small amount of catalyst (a mixture of potassium sulphate, copper sulphate, selenium, in the ratio of 32:5:1) into digestion flasks and then 1 ml aliquots of concentrated sulphuric acid was added. The mixture was heated for 2-3 hours. After the digest become clear, the flask was then allowed to cool.

After cooling, the digested mixture was diluted to about 100 ml by washing the contents of the flask three times with deionized water. The dilution was then transferred to a Markham still with 4 ml of 40% sodium hydroxide. The flask was carefully brought to boil and the distillation continued to remove the ammonia, which was collected in 10 ml of saturated boric acid. The boric acid was then titrated against standard 0.01N HCl, using Tashiro's indicator (methyl red, 2.0 g; methylene blue, 1 g; absolute alcohol, 1 litre).

The crude protein was then calculated by multiplying the nitrogen values by the constant factor 6.25 (Markham 1942).

2.7.2 Extraction of indole-3-acetic acid (IAA)

(i) Extraction of IAA from Agaricus bisporus fruit bodies and mycelium

Methods were modified from those adopted by Leopold

et. al. (1953), Larsen (1955 a, b), Leopold (1955 b), Bentley (1961 a), Audus (1972 a), Roberson, et. al. (1976), Tillberg (1974), Hillman (1978).

Samples were homogenized and covered with redistilled methanol (100%), using 4 ml for each original gram fresh weight of material. The mixture was stored at 4°C for 24 hours, filtered through a fine nylon sheet and the residue resuspended in cold methanol (2 ml/g original fresh weight), stored for 2-3 hours and filtered through the same filter. The tissue was washed three times with cold methanol (10-15 ml). Methanolic extracts were combined and centrifuged at 3000 g for 30 minutes and reduced to one tenth volume (mainly aqueous) at 35-40°C in a rotary evaporator. The aqueous fraction was centrifuged at 10000 g for one hour and adjusted to pH 2.5-3 using 2N HCl before further purification (section 2.8.1).

(ii) Extraction of IAA from culture filtrates

Filtrates from fungal cultures were prepared by filtering through Whatmann No. 1 filter paper. Filtrates from bacteria culture were prepared by centrifuging at 5000-6000 g for 30-40 minutes, and collecting the supernatant.

Filtrates were extracted, according to a modifi-

cation of methods used by Thimann (1935) and Mace (1965), with peroxide-free diethyl ether (Larsen, 1955 a). Thus, the filtrate was adjusted to pH 2.5-3.0 and extracted three times with equal volume of peroxide-free diethyl ether. The etheral fractions were combined and stored at -20°C for 12 hours, then filtered using Buchner filtration. The filtrate was then reduced in volume to a residue which was taken up in 1 ml methanol (100%) in preparation for paper chromatography.

(iii) Extraction of IAA from compost & casing soil

Samples from compost and casing soil (100 g fresh weight) were homogenized and extracted with 300-400 ml of 80% methanol and stored for 24 hours at 4°C. The mixture was filtered through a fine nylon sheet. The residue was resuspended in 300 ml of 80% methanol, stored for 4-5 hours at 4°C and filtered through the same filter. The filtrates were combined and centrifuged at 3000 g for one hour then reduced in volume at 35-40°C in a rotary evaporator. The aqueous fraction was then centrifuged at 10000 g for one hour and adjusted to pH 2.5-3 using 2N HCl in preparation to further purification (section 2.8.1).

2.7.3 Extraction of gibberellins

(i) Extraction of gibberellins from Agaricus bisporus fruit bodies and mycelium

The methods were modifications of those adopted by Pegg (1973), and Hillman (1978), using methanol as an extractant. Samples were homogenized and covered with an excess of cold methanol. The mixture was stored at 4°C for 24 hours, filtered through a fine nylon sheet, and the residue was resuspended in cold methanol (3 ml for each original gram fresh weight), stored at 4°C for 4-5 hours and filtered through the same filter. The methanolic extracts were combined, centrifuged at 3000 g for 40 minutes and reduced to the aqueous phase in a rotary evaporator at 35-40°C. The aqueous fraction was diluted at least two-fold with 0.5 M. phosphate buffer and adjusted to pH 8.0-8.2 before further purification.

(ii) Extraction of gibberellins from culture filtrates

The procedure used for gibberellin extraction was a modification to methods used by Pegg (1973) and Hillman (1978), using ethyl acetate as an extractant for gibberellins. The filtrates of Agaricus bisporus culture (or other micro-organisms) prepared as in sections 2.3 and 2.5.2 was adjusted to pH 8.2 using phosphate buffer. More impurities were removed from the aqeuous

fraction by partition (three times) against equal volumes of peroxide-free diethyl ether. The aqueous phase was then adjusted to pH 2.5-3 and extracted five times with half volume of ethyl acetate. The ethyl acetate fraction was stored at -20°C for 12 hours filtered through a Buchner funnel and evaporated at 30°C. The residue was then taken with 1 ml of methanol for later use in paper chromatography.

(iii) Extraction of gibberellins from compost and casing soil

Samples (100 g fresh weight) from compost and casing soil were extracted with 300-400 ml of 80% methanol according to a method used by Pegg (1973). The mixture was then stored at 4°C for 24 hours, filtered through a fine nylon sheet, the material residue resuspended in 300 ml of 80% methanol and filtered through the same filter. The methanolic extracts were collected, centrifuged at 3000 g for 40 minutes and evaporated to the aqueous phase in a rotary evaporator at 35-40°C. The aqueous phase was centrifuged at 10000 g for one hour and adjusted with phosphate buffer to pH 8-8.2 before further purification.

2.7.4 Extraction of cytokinin

(i) Extraction of cytokinin from Agaricus bisporus fruit bodies and mycelium

The methods used by Letham (1974), Letham et. al.

(1969), Audus (1972 c), and Hillman (1978), were adopted, using ethanol as an extractant. Samples from fruit bodies or mycelium were cut into small pieces, macerated with 90% ethanol and stored at 4°C for 12 hours. The mixture was filtered through a fine nylon sheet, residues re-extracted with 70% ethanol at 0°C for 3-4 hours, filtered through the same filter. The ethanolic extracts were combined and centrifuged at 3000-4000 g for 40 minutes. The supernatant was reduced to the aqueous phase in a rotary evaporator at 30-40°C, and centrifuged at 10000 g for one hour. The supernatant was then subjected to a clean-up procedure (Letham, 1974) by partitioning three times with equal volume of ethyl acetate at pH 2.5. The aqueous phase was then collected and adjusted to pH 8.2 in preparation for further purification.

(ii) Extraction of cytokinin from culture filtrates

The filtrates from static liquid cultures of Agaricus bisporus (or other micro-organisms) was firstly subjected to a clean-up procedure (Letham, 1974), by partitioning three times with equal volumes of ethyl acetate at pH 2.5. The aqueous phases were collected and adjusted to pH 8.2, extracted three times with double volume of ethyl acetate and evaporated. The material residue was taken up with 1 ml of methanol before further purification.

(iii) Extraction of cytokinin from compost and casing soil

Samples (100 g fresh weight) from compost or casing soil were extracted with 400 ml of 80% ethanol at $0^{o}C$ for 24 hours, filtered through a fine nylon sheet, and the material residue was re-extracted with 300 ml cold 70% ethanol, then filtered through the same filter. The ethanolic extracts were combined and centrifuged at 3000-4000 g for 40 minutes. The supernatant was reduced to the aqueous phase at 30-$40^{o}C$ in a rotary evaporator and centrifuged at 10000 g for one hour. The supernatant was subjected to the usual "clean-up" procedure to remove impurities. Thus the aqueous phase was partitioned against the equal volume of ethyl acetate (three times) at pH 2.5, and the aqueous fraction adjusted to pH 8.2 before further purification.

2.8 Purification of phytohormone extracts using solvent partition and chromatographic techniques

2.8.1 Purification of IAA

The aqueous phase prepared as in section 2.7.2 was purified using the following techniques.

(i) Partition between solvents

The aqueous phase adjusted to pH 2.5-3 was partit-

ioned three times against equal volumes of peroxide-free diethyl ether (freed from peroxide with ferrous sulphate and calcium oxide and redistilled according to Larsen, 1955 a). The etheral fractions were combined, stored at -20°C overnight, the ice separated by filtration and rejected. The filtrate was evaporated to dryness under reduced pressure. The extract residue was taken up in 1 ml methanol in preparation for the next purification step.

(ii) DEAE cellulose column chromatography

Extracts from fruit bodies (flat stage) and lamellae, which contain pigments which interfere with thin layer and paper chromatographic techniques, were purified by DEAE cellulose column chromatography before application of thin layer and paper chromatographic techniques (Woodruffe et. al. 1970).

The column was prepared as follows: 15 g of diethylaminoethyl (DEAE) - cellulose (Whatman DE 1), previously treated successively with 0.5N HCl, water, 0.5 N NaOH and washed with water until a final pH of 6.5-7.5 was reached. The column was then loaded with the extract obtained from previous step (partition between solvents) and eluted with 300 ml of distilled water to remove unbound materials and then discarded. 300 ml

of 0.05 M sodium sulphate solution was then used to elute the column to remove the ionically bound substances including IAA. The sodium sulphate used to elute the column was collected and acidified to pH 2.5-3 and partitioned three times against equal volumes of diethyl ether. The etheral fractions were combined, stored at -20°C, filtered and reduced to a residue at 30°C in a rotary evaporator. The residue was taken up in 1 ml methanol for further chromatography.

(iii) Thin-layer chromatography (TLC)

The extract (after solvent partition or column chromatography) was applied as spots or streaks on one dimentional thin-layer chromatography (20 x 20 cm) carried 0.1 mm thick layer cellulose (Merck: DC Fertigplatten F) following Smith (1960), Sagi (1969), Woodruffe et. al.(1970) and Hillman (1978).

Plates were developed over 10 cm, with two solvent systems, namely:

1. Isopropanol: 35% ammonia : water (10:1:1, v:v:v);
2. Methyl acetate : isopropanol : 35% ammonia (45:35:20, v:v:v).

Subsequently, the plates were dried in darkness

for 10 to 20 minutes in a stream of cold air before testing.

(iv) Paper chromatography (PC)

The extracts (after solvent partition or column chromatography) were streaked on Whatman 3 MM filter paper. Chromatograms were developed with the same solvent system used for TLC. The chromatographic solvent was allowed to run for 15 cm. The papers were then dried in darkness before identification and biological assay.

2.8.2 Purification of gibberellin extract

The aqueous fraction prepared in section 2.7.3 was purified using the following techniques.

(i) Partition between solvents

The aqueous fraction adjusted to pH 8.2 (see section 2.7.3) was partitioned three times against peroxide-free diethyl ether to remove more impurities. The aqueous fractions were combined, adjusted to pH 2.5-3, and partitioned five times with half volume of ethyl acetate. The ethyl acetate fractions were then combined, stored at -20^{o}C overnight, then filtered through a Buchner funnel and evaporated at 30^{o}C to the residue. The residue was taken up with 1 ml of methanol for paper

chromatography.

(ii) Paper chromatography

The extract (after solvent partition) was streaked on Whatman 3 MM filter paper. Also a given amount of synthetic gibberellic acid was streaked on the same paper, served as reference. Chromatograms were developed with isopropanol : ammonia : water (10:1:1, v:v:v) and the solvent was allowed to run for 15 cm. The paper was then dried in darkness for 20 minutes before identification and bioassay.

2.8.3 Purification of cytokinin extracts

The aqueous fractions prepared in section 2.7.4 were purified as follows:

(i) Partition between solvents

The aqueous fraction adjusted to pH 8.2 was partitioned three times with double volume of ethyl acetate, stored at -20°C overnight, filtered, and the filtrate reduced in volume, at 30°C, to a residue. The residue was taken in 1 ml methanol for use in paper chromatography.

(ii) Paper chromatography

The extract (after solvent partition) was streaked on Whatman 3 MM filter paper (after washing with methanol and drying). A given amount of synthetic

kinetin was also streaked on the same paper which was developed with n-butanol : formic acid : water (10:4:5, upper phase), following the methods of Letham et. al. (1969). The chromatographic solvent was allowed to run for 15 cm. The paper dried for 20-30 minutes in a stream of cold air in preparation for a bioassay.

2.9 Identification of phytohormones

2.9.1 Identification of IAA

(i) Chemical test

The following chemical reagents were used to determine the presence of IAA in extracts:

The Salkowski's reagent

The Salkowski's reaction test was carried out using a modification of Tang and Bonner's (1947) ferric chloride reagent (a mixture of 0.5M $FeCl_3$, 300 ml of H_2SO_4, sp.gr 1.84, 500 ml of distilled water). The reagent was sprayed on the developed chromatograms. The chromatograms were dried in a stream of cold air for 20-30 minutes. The characteristic red colour for IAA was developed after drying.

The Ehrlich's reagent

A modification of Ehrlich's reagent was sprayed on chromatograms following Harley, Mason and Archer (1958).

The reagent was prepared by dissolving 1 g of p-dimethylaminocinnamaldehyde in a 50:50 mixture of ethanol and 6N HCl. After drying the chromatograms, the characteristic colour was developed when IAA present.

A colorimetric estimation of IAA was carried out using another modification of Ehrlich's reagent (Anthony and Street, 1970). Ehrlich's reagent was prepared by dissolving 2 g of p-dimethylaminobenzaldehyde in 100 ml of 2.5N HCl. Two ml of this reagent was added to the standard reaction mixture which consisted of 1 ml of standard solution of IAA and 2 ml of trichloracetic acid (100% w/v). After 30 minutes, the colour intensity and absorption spectra of the colour complexes were measured in Unican SP800 recording spectrophotometer.

(ii) Physico-chemical test

The R_F-values (the ratio of the distance moved by the molecule concerned to the distance moved by the solvent front) of synthetic IAA applied on thin-layer and paper chromatograms were calculated after locating the spot by spraying the chromatograms with Salkowski or Ehrlich's reagents. These compared with R_F-values obtained from spots developed by endogneous IAA which is

present in the extract applied on the same chromatograms.

(iii) Biological activity

Pea root bioassay

This procedure was carried out according to Leopold (1955 b), using pea roots as bioassay materials. After developing and drying the paper chromatograms, the zone suspected to contain auxin was located by cutting off a strip spotted with synthetic IAA and then sprayed with Salkowski or Ehrlich's reagent. The auxin occurring at the R_F of synthetic IAA was removed by cutting a strip of the paper chromatograms (3 cm x 3 cm), placed in a Petri plate containing 10 ml of nutrient solution (containing 0.0025 M $CaSO_4$ and 1% sucrose in deionized water) in preparation for the bioassay materials.

For preparing the pea roots for the test, the seeds of garden peas (Pisum sativum) were placed between two filter papers moistened with deionized water, and incubated at 25°C for 4 days. Subsequently, roots were carefully selected for straight, thin, uniform appearance, and then 5 mm pieces were cut (including the root tip). Ten of these pieces were transferred to each Petri plate, and arranged in rows on the strip of the

paper chromatogram. The control was similarly prepared, but it was supplied with a strip removed from an unloaded paper chromatogram developed in the same tank.

The references of the bioassay were prepared as mentioned above using five strips removed from chromatograms loaded with five concentrations of synthetic IAA (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}M).

All the plates were incubated at 25°C for 24 hours, and then the root length was measured. The mean of 10 replicates was calculated and the elongation of auxin treated roots was expressed as percentages of the elongation of the control.

Cress root bioassay

Cress seeds (Lepidium sativum) were germinated on filter papers moistened with deionized water, incubated at 27°C for 24 hours, and the seedlings with radical 5 mm long were slected as test plants (see Audus and Thresh, 1953).

Locating auxin on developed paper chromatograms was carried out as in previous test (pea root test). Thus the strips of paper chromatograms were placed in the Petri plates, each containing 5 ml of deionized water.

10-15 seedlings were transferred to each plate and arranged in rows on the paper chromatogram, while the reference was prepared by using many strips from paper chromatograms loaded with different concentrations of synthetic IAA. All plates were incubated at 27°C for 24 hours, and then the root length was measured. The mean of 10-15 replicates was calculated as well as the rate of growth inhibition. Thus a standard curve of synthetic IAA was constructed and used for calculation of the amount of auxin in the tested extracts (in terms of IAA equivalents).

2.9.2 Identification of gibberellins

Crude extracts of many plants species and culture filtrates of some fungi and bacteria have been found to possess biological properties similar to the gibberellins. Several workers attempted to identify the compound responsible by paper chromatographic comparison and using one or more gibberellin bioassay (Jones, et.al. 1963; Frankland et. al., 1960; Pegg, 1966; Adler, et. al., 1973). Thus, the significant growth promotion in the plant treated with the extract indicates that this extract possess gibberellin-like activity. The amount of this activity can be determined by comparing the growth promotion with that caused by known amounts of GA_3.

Identification of gibberellins, throughout the present research, was carried out using paper chromatography and the lettuce hypocotyl bioassay.

Lettuce hypocotyl bioassay

This test was carried out according to Frankland and Wareing (1960), using two-day old seedlings of lettuce (Lactuca sativa, variety: Grand Rapid). Lettuce seeds were first germinated on filter papers moistened with deionized water and incubated in the dark at 27°C for two days. Seedlings with similar radicals (6-8 mm long) were then selected and transferred to the bioassay plates. Petri plates lined with filter papers moistened with 6 ml of deionized water served as bioassay plates. To each plate a chromatogram strip was transferred. The strip was prepared by cutting the developed paper chromatogram to longitudinal portions, each loaded with known amounts of the extract or GA_3. Each portion of the paper chromatogram was cut into five transverse strips. Three different concentrations of the extract (100 µl , 300 µl , 500 µl) were applied on the paper chromatogram, while five concentrations of synthetic gibberellic acid (5 µg, 10 µg, 15 µg, 20 µg, 25 µg), were also applied on other paper chromatograms and developed in the same tank, for constructing the standard curve.

Ten seedlings were then transferred to each plate and arranged in rows on the chromatogram strip. The control was similarly prepared by using a strip removed from an unloaded paper chromatogram. All the plates were then incubated in an illuminated incubator at 27°C for five days. The length of hypocotyl was measured and the mean of 10 replicates was calculated. The elongation of extract and gibberellic acid treated hypocotyl was expressed as percentages of the elongation of control. By using the standard curve and correlation test the amount of gibberellins in the extract was calculated in terms of gibberellic acid equivalents.

2.9.3 Identification of cytokinins

Radish cotyledons bioassay

This test was carried out according to Letham (1971) using seeds of radish (Raphanus sativus L.cv. Long Scarlet). Seeds were selected for uniformity, germinated on filter papers moistened with deionized water, and incubated in the dark at 25°C. After 24 hours the smallest cotyledons were removed from the seedlings and transferred to the bioassay plates. Each bioassay plate was prepared by placing one circle of filter paper in a Petri plate (9 cm) and then 3 ml of phosphate buffer were added. Also, one strip of developed paper

chromatogram (see section 2.8.3) loaded with a known amount of the extract or synthetic kinetin was transferred to each plate.

The extract was applied on the paper chromatogram at three different concentrations (100 µl, 300 µl, 500 µl), while the synthetic kinetin (reference) was applied at five different concentrations (5 µg, 10 µg, 15 µg, 20 µg, and 25 µg). The paper chromatogram was cut into several longitudinal strips and then each one was cut into five transverse strips. Each transverse strip was transferred to one bioassay plate, and then 8-10 cotyledons were arranged in rows on it. The control was similarly prepared using a strip removed from an unloaded paper chromatogram.

All the bioassay plates were incubated at 25°C in an illuminated incubator. After three days, the cotyledons were blotted dry and weighed. The mean of 10 replicates was calculated and the increments in the weight of the treated cotyledons was expressed as percentage of the cotyledon weight of the control. Subsequently, standard curve and correlation test were used to calculate the amount of cytokinin in the extract in terms of kinetin equivalents.

SECTION 3

EXPERIMENTAL

PART 1 PHYTOHORMONES AND VEGETATIVE GROWTH:

3.1 Growth on semi-solid agar in Petri plates

Laboratory cultures of A.bisporus are normally maintained on semi-solid media, but for the estimation of growth the method of measuring extension growth of the developing colony is known to be disadvantageous, since estimates of growth do not necessarily reflect growth in terms of biomass. However, when supplemented with arbitrary assessments of colony density the accuracy of agar techniques are greatly improved. Also, agar techniques allow for other observations such as the occurrence of sectoring, known to be of relevance in the productivity of A.bisporus. Colony form may be influenced by treatments applied in other ways, especially the regularity of colony form and extension. In addition, growth on agar allows microscopic examination of individual hyphal cells.

The methods adopted in this study conformed to a regular pattern irrespective of phytohormone treatment. This involved preparing a semi-solid synthetic agar medium outlined in section 2.2.2. Phytohormones were prepared in solution and filter-sterilized (section 2.2.3) and added to autoclaved media at various concentrations, before solidification.

After inoculation plates were incubated at the optimum temperature for A.bisporus (25°C) and radial growth of colonies were measured at convenient time intervals, normally after 14 days and 28 days growth. Normally six replicates were prepared for each treatment and means calculated and differences were determined to be significant or not significant, according to statistical techniques given in Appendix 5.1.

At each growth measurement, the density of colonies and the frequency of colony sectoring were assessed on an arbitrary scale and where relevant ,were recorded.

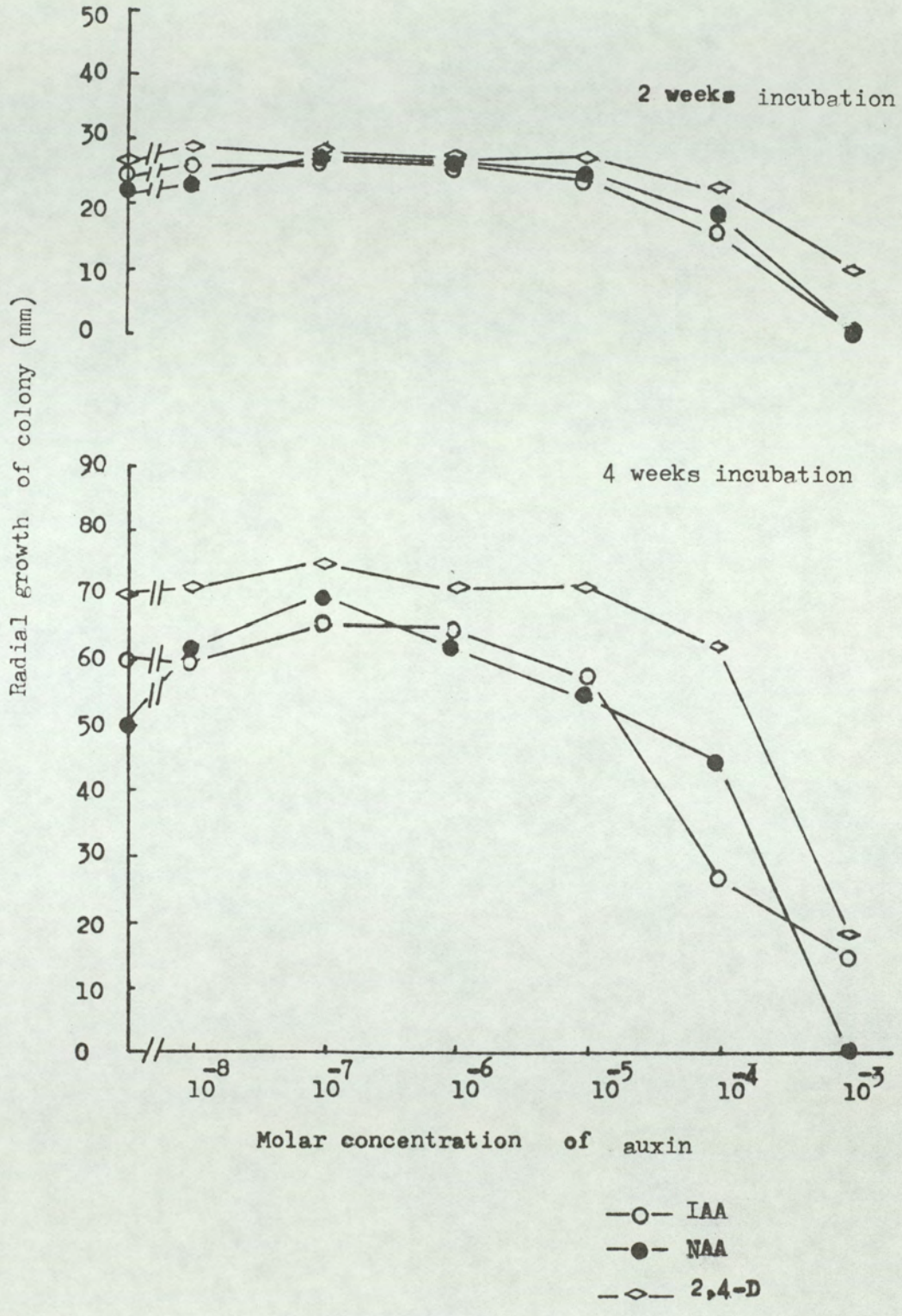
Plates were also examined using Phase-contrast microscopy and any abnormal cellular development was noted. A more detailed study of the elongation of individual hyphal cells noted at some levels of phytohormone application was also undertaken.

3.1.1 Auxins

The results of adding the three known auxins IAA, NAA and 2,4-D to the synthetic medium are recorded graphically in Figure 3.1 and tabulated in Appendices 1.1, 1.2, and 1.3.

After two weeks incubation, there was no marked

Figure 3.1 Effects of auxins on mycelial growth on semi-solid agar :



influence on growth rate except for a significant inhibition of growth at concentrations higher than 10^{-5}M. This concentration effect was evident after four weeks incubation. However IAA completely inhibited growth up to two weeks of incubation, but growth was restored after four weeks, suggesting a possible inactivation of excess IAA, by A.bisporus mycelium.

Both IAA and NAA significantly stimulated growth after four weeks of incubation when included at a concentration of 10^{-7}M. No marked stimulation occurred with 2,4-D.

Table 1.1 summarizes the effects of auxins on density, sectoring and shape of the colony. Density and shape of colony were greatly influenced by increasing concentrations of IAA, while sectoring frequency was only increased at concentrations of 10^{-5} to 10^{-7}M. (Plates 1.1, 1.2) The same pattern also resulted from the additions of NAA and 2,4-D.

3.1.2 Gibberellin

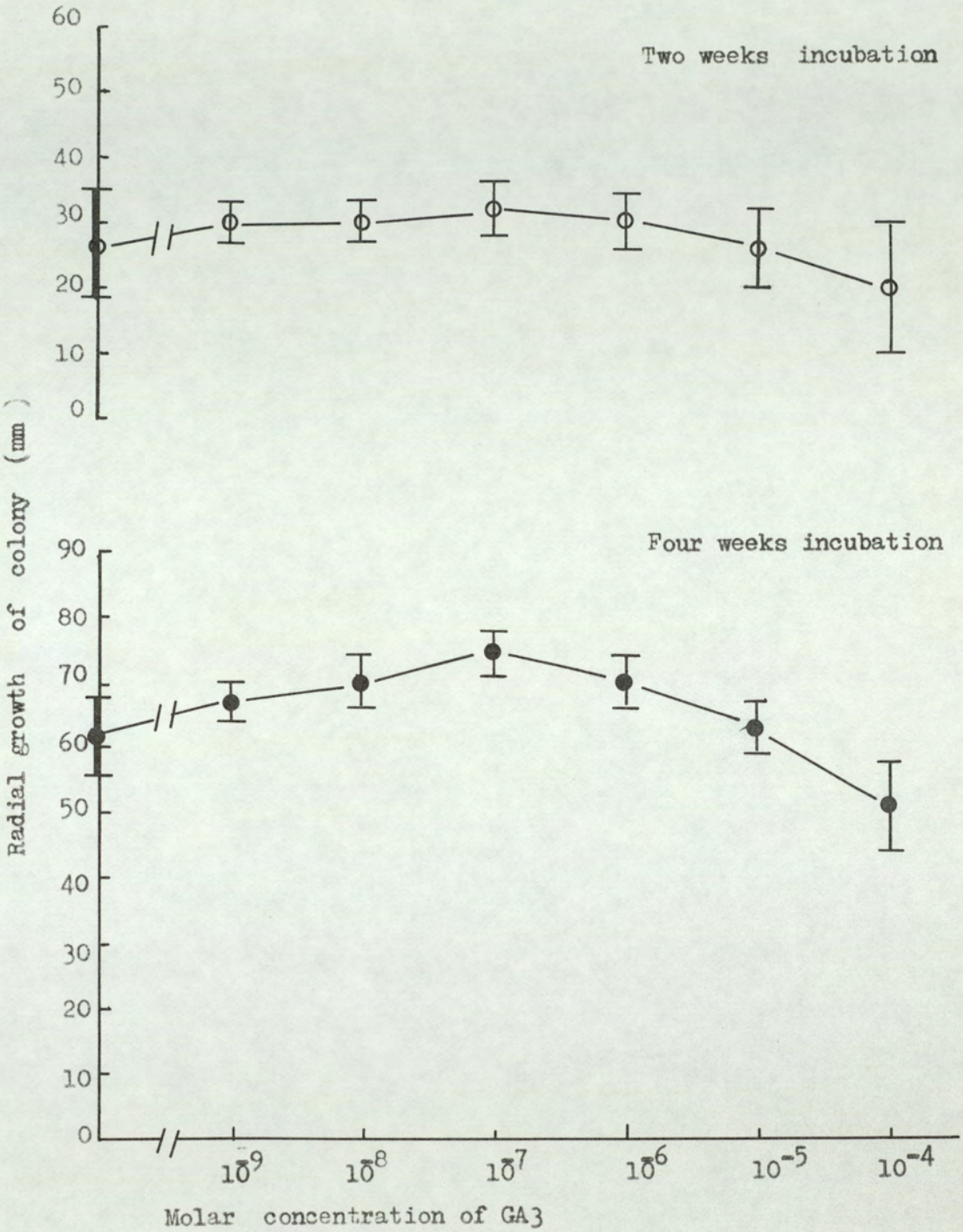
The pattern of growth after gibberellin addition (GA_3) over the range of concentrations, was similar to that obtained from auxin addition (Figure 3.2). There was no inhibition of growth at the highest concentration tested (10^{-4}M) and growth differences were

Table 1.1 The effects of auxins on colony form of A.bisporus

Auxins		Concentration applied (M)						
		0	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
IAA	DC	+	+++	++	+	+	+	+
	SF	++	-	++	++++	++++	+++	++
	SHC	+++	+	+	+++	+++	+++	+++
NAA	DC	+	-	++	++	+	+	+
	SF	+	-	++	++++	++++	++++	++
	SHC	+++	-	++	++	+++	+++	+++
2,4-D	DC	+	++	++	++	+	+	+
	SF	++	-	++	+++	+++	++++	++
	SHC	+++	+	++	+++	+++	+++	+++

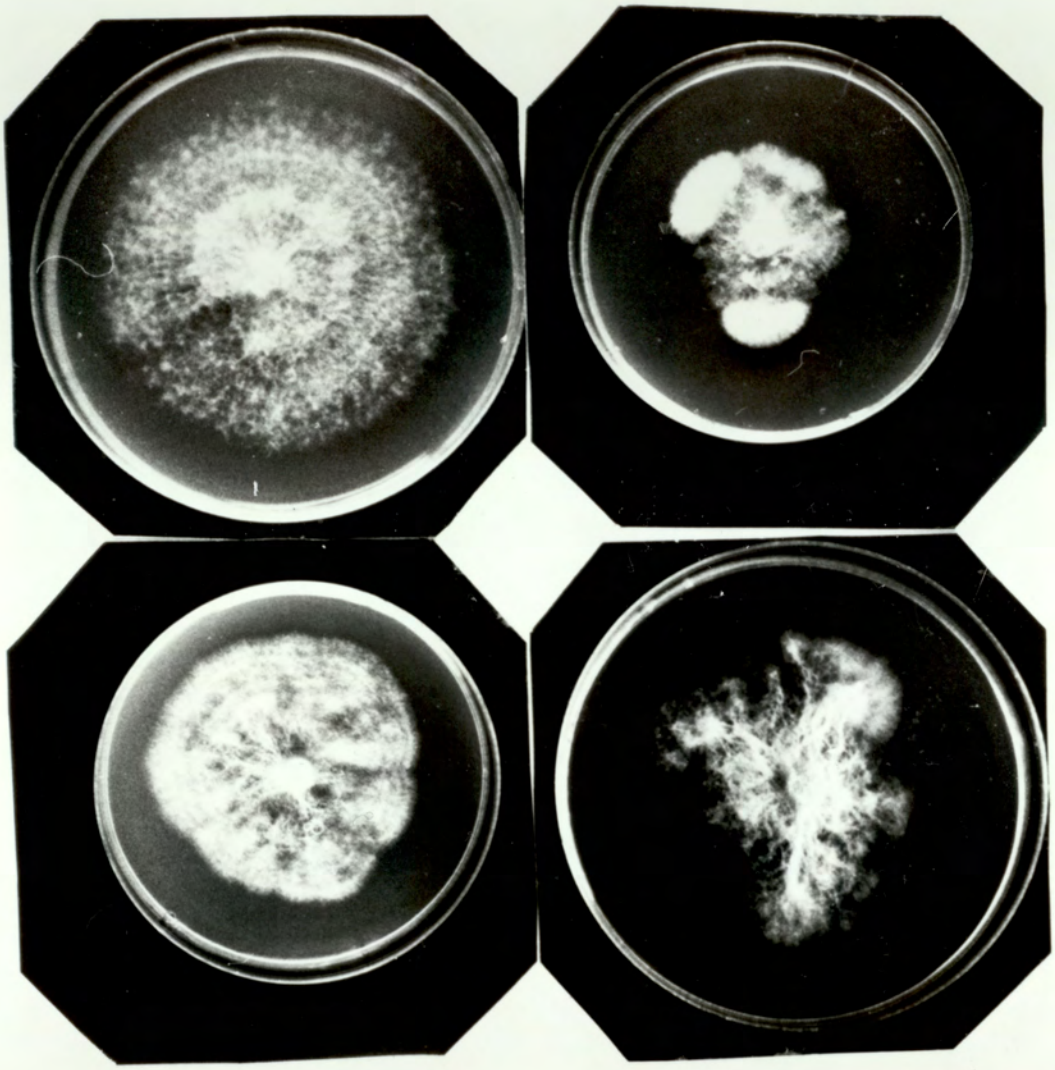
DC = Density of colony. + = normal density; ++ = moderately dense colony; +++ = dense colony; - = no growth.
SF = Sectoring frequency. + = rare; ++ = few; +++ = many; ++++ = intense; - = no sectoring.
SHC = Shape of colony. + = regular shape; ++ = slight irregular; +++ = irregular; - = no growth.

Figure 3.2 Effect of GA3 on mycelial growth on semi-soild synthetic medium in Petri plates .



Note :Vertical bars are the confidence limits of the means at p=0.05 .

Plate. 1.1 Effect of phytohormones on colony shape .



1. control (without phytohormoe addition).
2. IAA treatment (10^{-4}M).
3. GA_3 treatment (10^{-4} M).
4. K treatment (10^{-4}M) .

more marked after four weeks of incubation. Significant stimulation of radial growth was obtained at 10^{-6} 10^{-7} and 10^{-8}M, but as with auxins, the optimum was at 10^{-7}M (Appendix 1.4).

Density of colony was increased at concentrations of 10^{-4} and 10^{-5}M while irregular colonies were only given at the highest concentration. Sectoring frequency was increased at concentrations of 10^{-5} and 10^{-6}M (Table 1.2, Plate 1.2).

Table 1.2 The effect of gibberellic acid on colony form of A.bisporus .

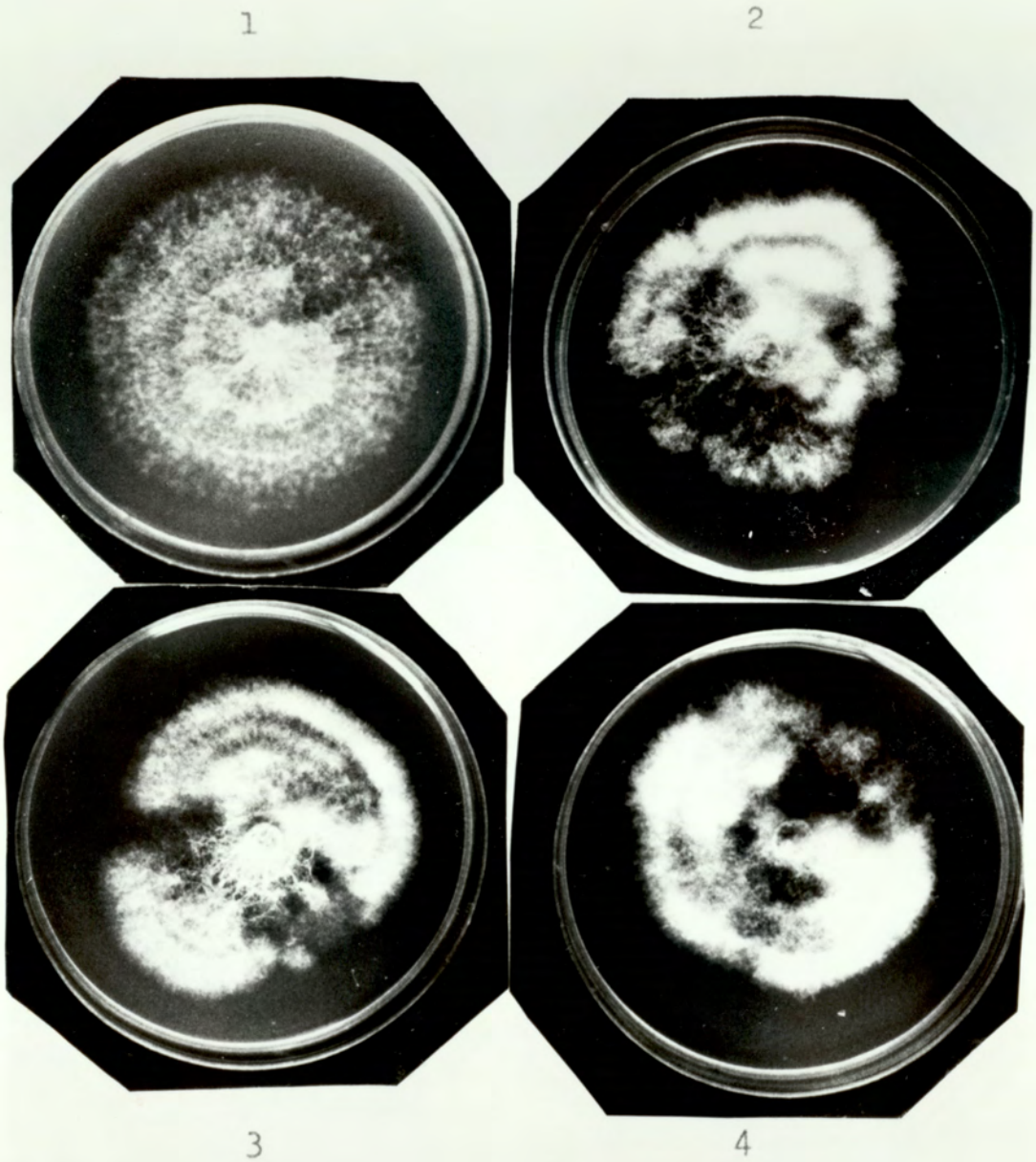
	concentration applied (mole)						
	0	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Colony density (DC)	+	++	++	+	+	+	+
Sectoring frequency (SF)	+	+	++++	+++	++	++	++
Shape of colony (SCH)	+	++	+	+	+	+	+

DC: Density of colony. + normal density; ++ moderately dense colony,

SF: Sectoring frequency. + rare; ++ few; +++ many; ++++ intense.

SHC: Shape of colony. + regular shape; ++ slightly irregular shape.

Plate 1.2 Effect of phytohormones on sectoring frequency.



1. Control (without phytohormone addition).
2. IAA treatment (10^{-6} M).
3. GA_3 treatment (10^{-5} M).
4. K treatment (10^{-6} M).

3.1.3 Cytokinin

The addition of kinetin to a semi-solid synthetic medium, resulted in growth stimulation at concentrations of 10^{-7} and 10^{-8}M but there was no inhibition of growth at the highest concentration tested 10^{-4}M (Figure 3.3 and Appendix 1.5).

A very pronounced effect on the form of colony was obtained (Plate 1.1). Sectoring of the colony was evident at concentrations of 10^{-6} - 10^{-9}M (Table 1.3). Regeneration of hyphal growth occurred at all points in the colony, giving a pronounced sectoring effect (Plate 1.2).

Table 1.3 The effect of cytokinin on colony form of A.bisporus

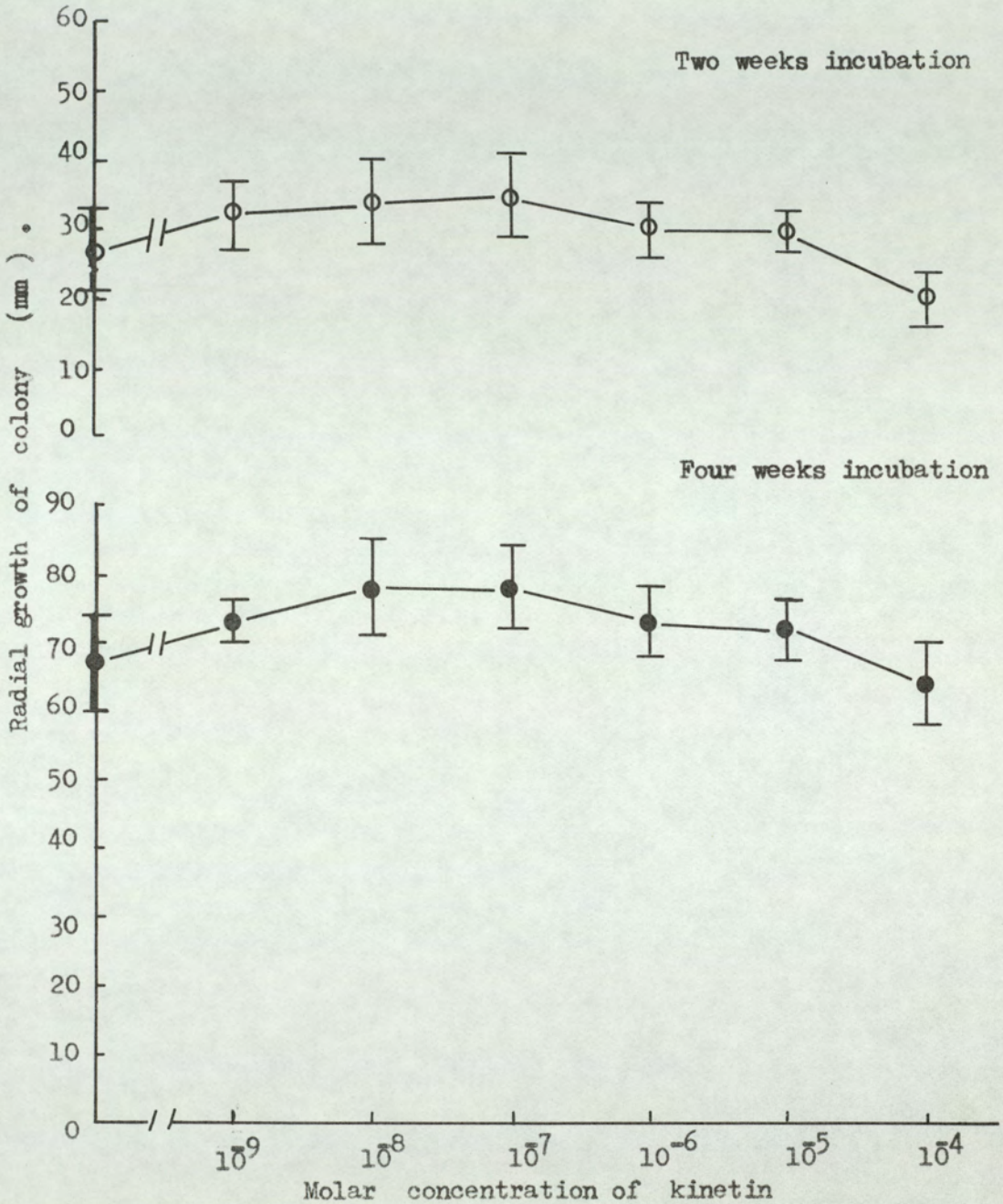
	Concentration applied (mole)						
	0	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{9}
Density of colony (DC)	+	++	+	+	+	+	+
Sectoring frequency (SF)	+	+	++	++++	++++	+++	+++
Shape of colony (SHC)	+	++	+	+	+	+	+

DC = Density of colony. + = normal density; ++ = moderately dense colony.

SF = Sectoring frequency. + = rare; ++ = few; +++ = many; ++++ = intense.

SHC = Shape of colony. + = regular; ++ = slightly irregular shape.

Figure 3.3 : Effect of kinetin on mycelial growth on semi-solid synthetic medium .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

3.1.4 Combination of indole-3-acetic acid with gibberellic acid, kinetin and gibberellic acid and kinetin

Since the phytohormones under study are known to interact in their action on growth and development in higher plants, their combined effects on A.bisporus in semi-solid medium were studied.

Various combinations of concentrations of IAA, GA_3 and K were tested and the results are reported in full in Appendices 1.6 and 1.7. The results from combinations of equal concentrations of IAA with GA_3 and kinetin are given in Figures 3.4 and 3.5.

Significant stimulation of growth occurred when IAA was combined with GA_3 at 10^{-5}M concentration and with kinetin at 10^{-7}Molar concentrations. This clearly indicates a significant balance between two phytohormones in inducing a stimulation of growth.

Similarly when combined in equal concentrations inhibitory concentrations were achieved at the high level of addition, but not when applied alone at the same concentrations.

Combinations of IAA, GA_3 and K at varying concentrations also influenced both stimulation of growth at low concentrations and inhibition of growth

Figure 3.4 :Effect of combined additions Of IAA and GA_3 on mycelial growth on semi-solid synthetic medium .

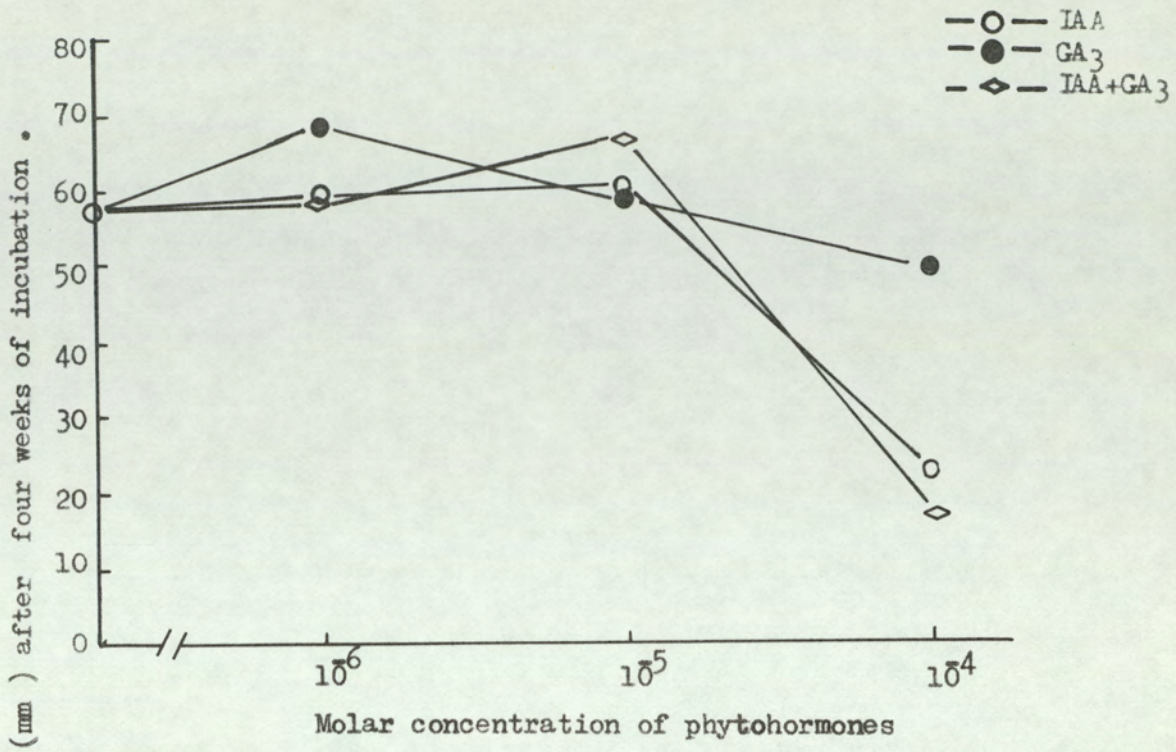
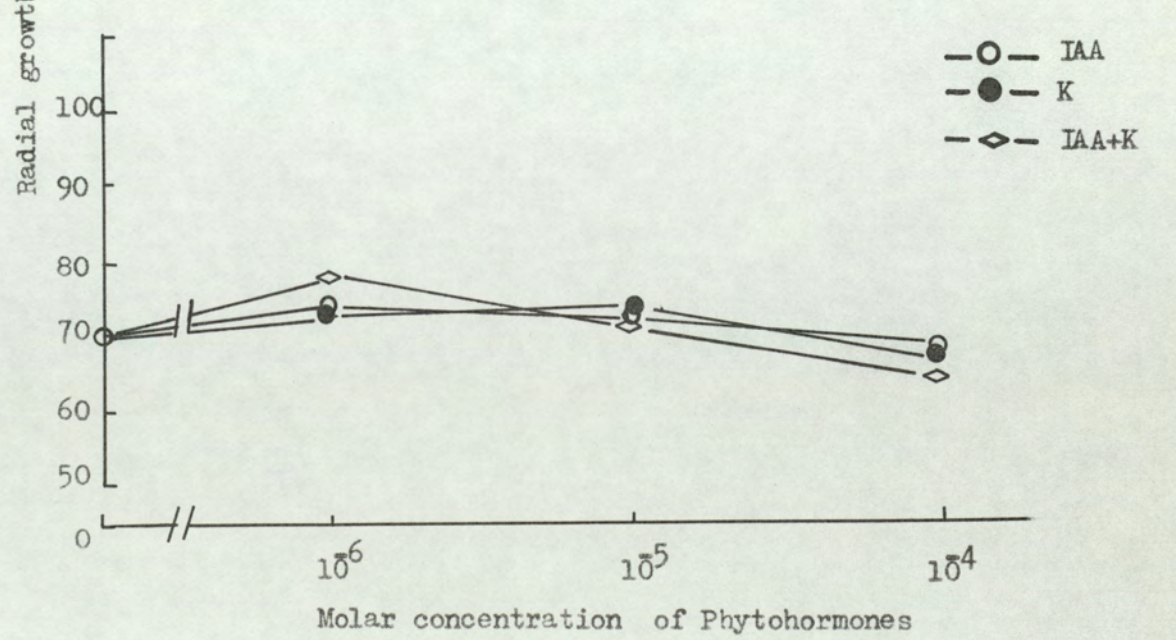


Figure 3.5 :Effect of combined additions of IAA &K on mycelial growth on semi-solid synthetic medium .



at high concentrations (Figure 3.6, Appendix 1.7) indicating again that the three phytohormones may interact with each other in controlling growth.

Sectoring frequency was also influenced by different concentrations (Appendices 1.6, 1.7, 1.8) but generally most concentrations of IAA with K increased sectoring frequency while most with GA_3 reduced sectoring.

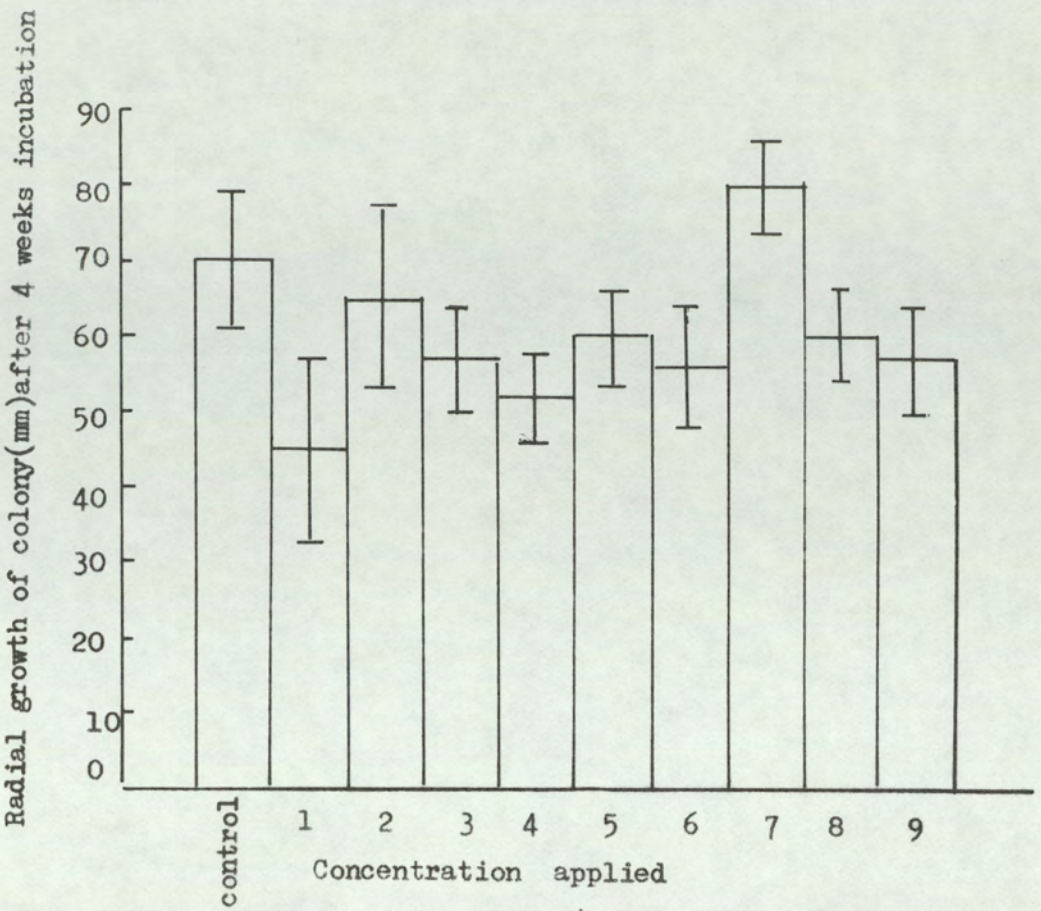
3.1.5 Phytohormones and hyphal cell characteristics

Semi-solid agar techniques permit observations on the effects of phytohormone treatments on cell characteristics. Since phytohormone effects in green plants are known to be active at the cellular levels, gross morphological changes in hyphal cell were observed using phase-contrast microscopy.

As in some green plants IAA and GA_3 influenced the size of hyphal cells of A.bisporus. In contrast, and in common with effects in green plants, kinetin decreased cell length. These characteristics are recorded in Figure 3.7 and tabulated in Appendix 1.9.

IAA significantly stimulated hyphal cell expansion over a wide range of concentrations (6×10^{-5}, 10^{-5}, 10^{-7}M) but maximum cell length was given at a concent-

Figure 3.6 :Effect of combined additions of IAA+GA_3+ K on mycelial growth on semi-solid synthetic medium .



1=Combined addition of IAA(10^{-4}M) +GA_3(6 X 10^{-5}M) + K (4 X 10^{-4}M) .

3=Combined addition of IAA (10^{-4}M) +GA_3(6X10^{-5}M)+ K (5X10^{-5}M) .

4=Combined addition of IAA (3X10^{-5}M) +GA_3(3X10^{-5}M) +K(4X10^{-4}M) .

5=Combined addition of IAA (6X10^{-5} M) +GA_3(3X10^{-5}M) +K(5X10^{-5} M) .

6=Combined addition of IAA(6X10^{-5}M)+GA_3(3X10^{-5}M)+ K (5X10^{-6}M)

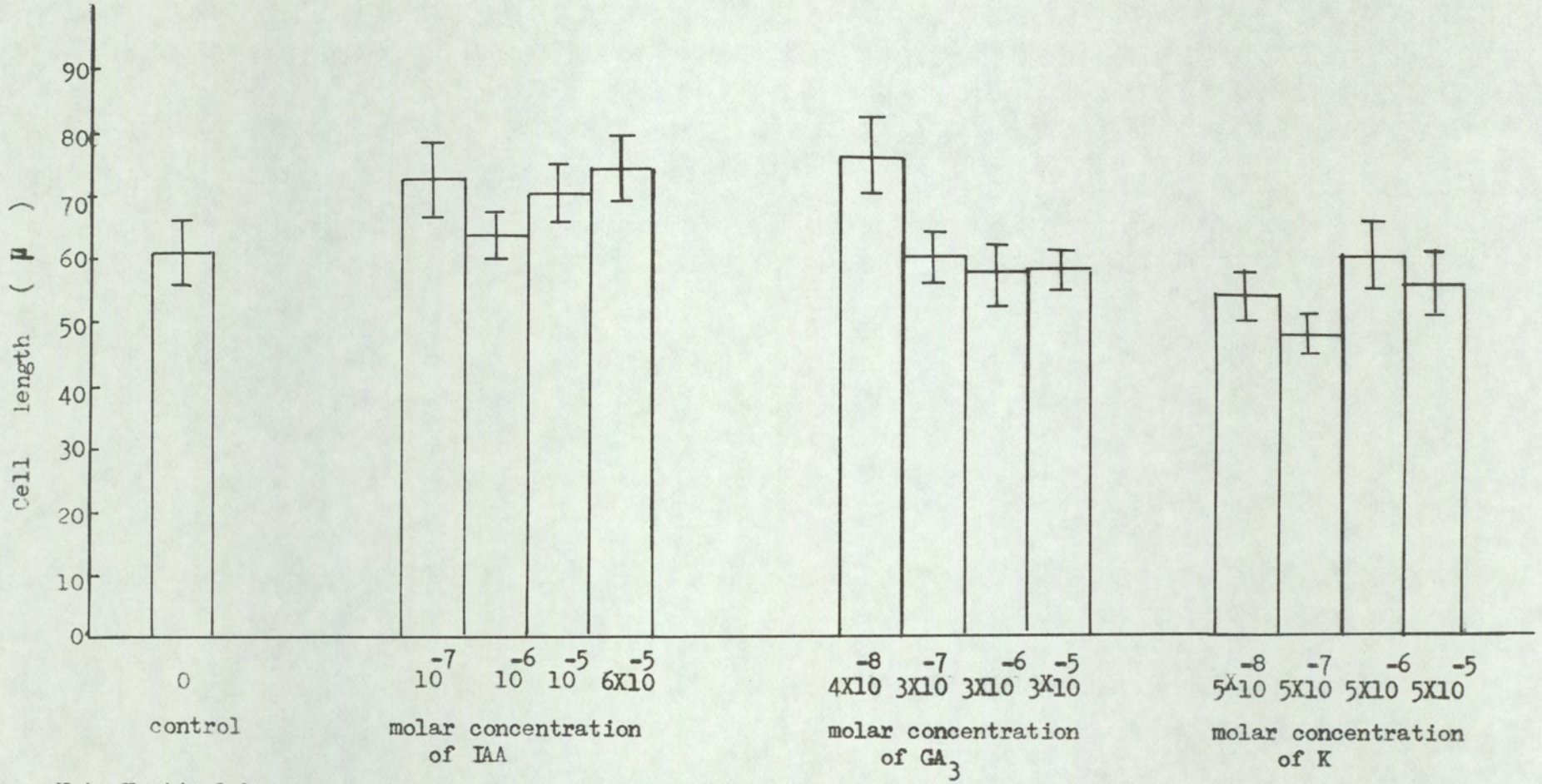
7=Combined addition of IAA(10^{-5}M) + GA_3 (3X 10^{-6}M)+K (5X10^{-6}M) .

8=Combined addition of IAA (10^{-5}M) + GA_3(3 X 10^{-6}M) + K (4X10^{-4}M) .

9=Combined addition of IAA (10^{-5}M) + GA_3(3X10^{-6}M) + K (5X 10^{-5}M) .

Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.7 :Effect of phytohormones on hyphal cell length on semi-solid synthetic medium .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

ration of 6 x 10^{-5}M. Gibberellic acid on the other hand stimulated cell extension at the lowest concentration tested viz 4 x 10^{-8}M. Kinetin decreased cell length at concentrations of 5 x 10^{-7} and 5 x 10^{-8}M.

3.2 Growth in liquid culture

Growth studies in liquid cultures permit a more accurate estimation of extent of growth than semi-solid Petri plates techniques, since growth is determined as dry weight. However, it is a destructive method and morphological observations are not normally possible. The methods adopted for liquid culture studies are outlined in section 2.3.

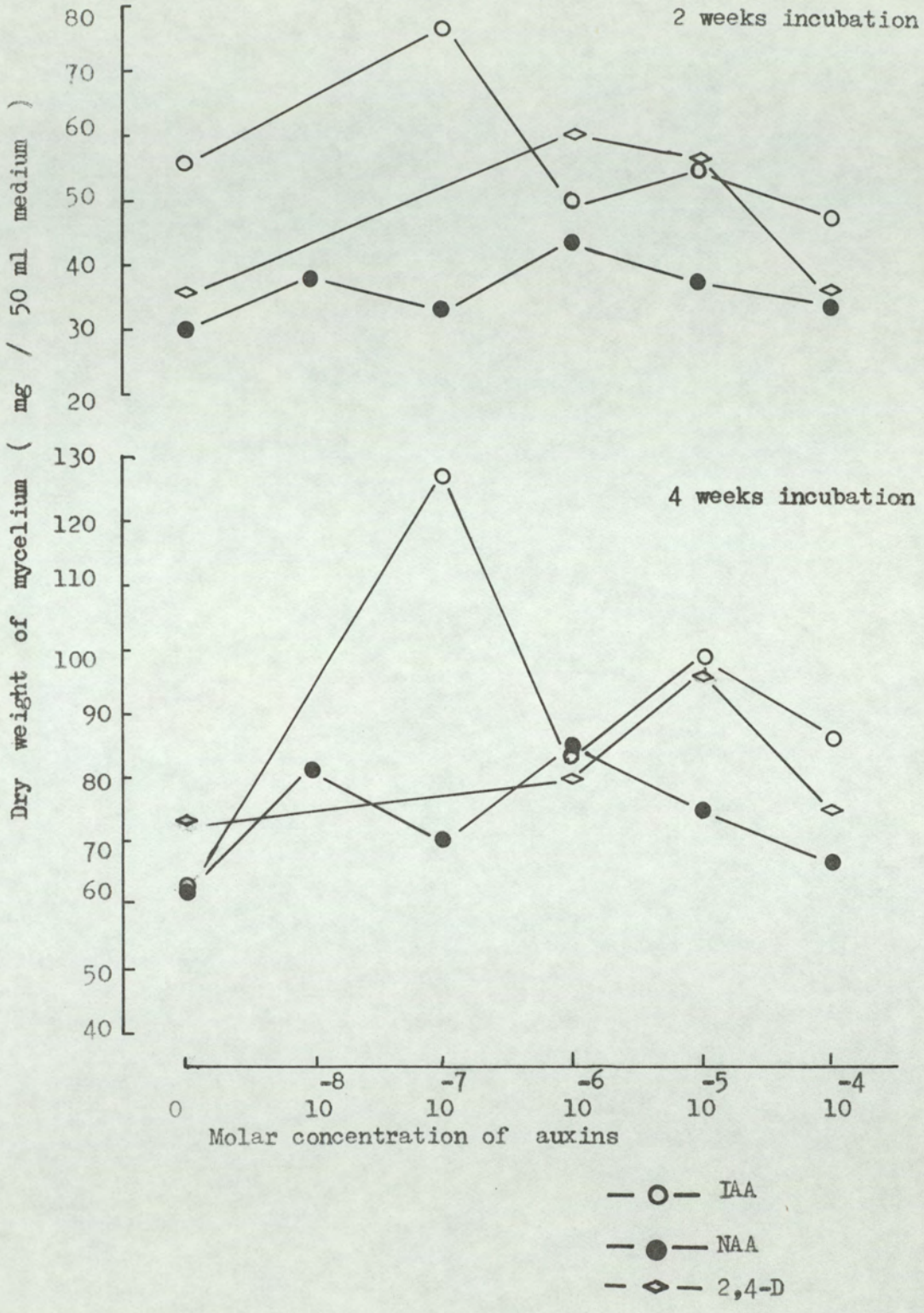
3.2.1 Auxins

After two weeks of incubation, dry weight was only increased at a concentration of 10^{-7}M (Figure 3.8, Appendix 1.10). After four weeks, a significant increase was obtained at all tested concentrations.

Similarly, the addition of NAA and 2,4-D slightly increased the dry weight after two weeks of incubation, but after four weeks all tested concentrations significantly increased mycelial dry weight.

In general, IAA was more effective in stimulating growth than both NAA and 2,4-D. For example, the optimum concentration of IAA (10^{-7}M) increased the dry

Figure 3.8 :Effect of auxins on mycelial growth in liquid culture .



weight by 108% while the optimum of NAA (10^{-6}M) and 2,4-D (10^{-5}M) increased the dry weight by 39% and 32%, respectively (Appendices 1.10, 1.11, 1.12).

In general, pH values were slightly influenced, especially when the growth was stimulated or markedly inhibited.

3.2.2 Gibberellin

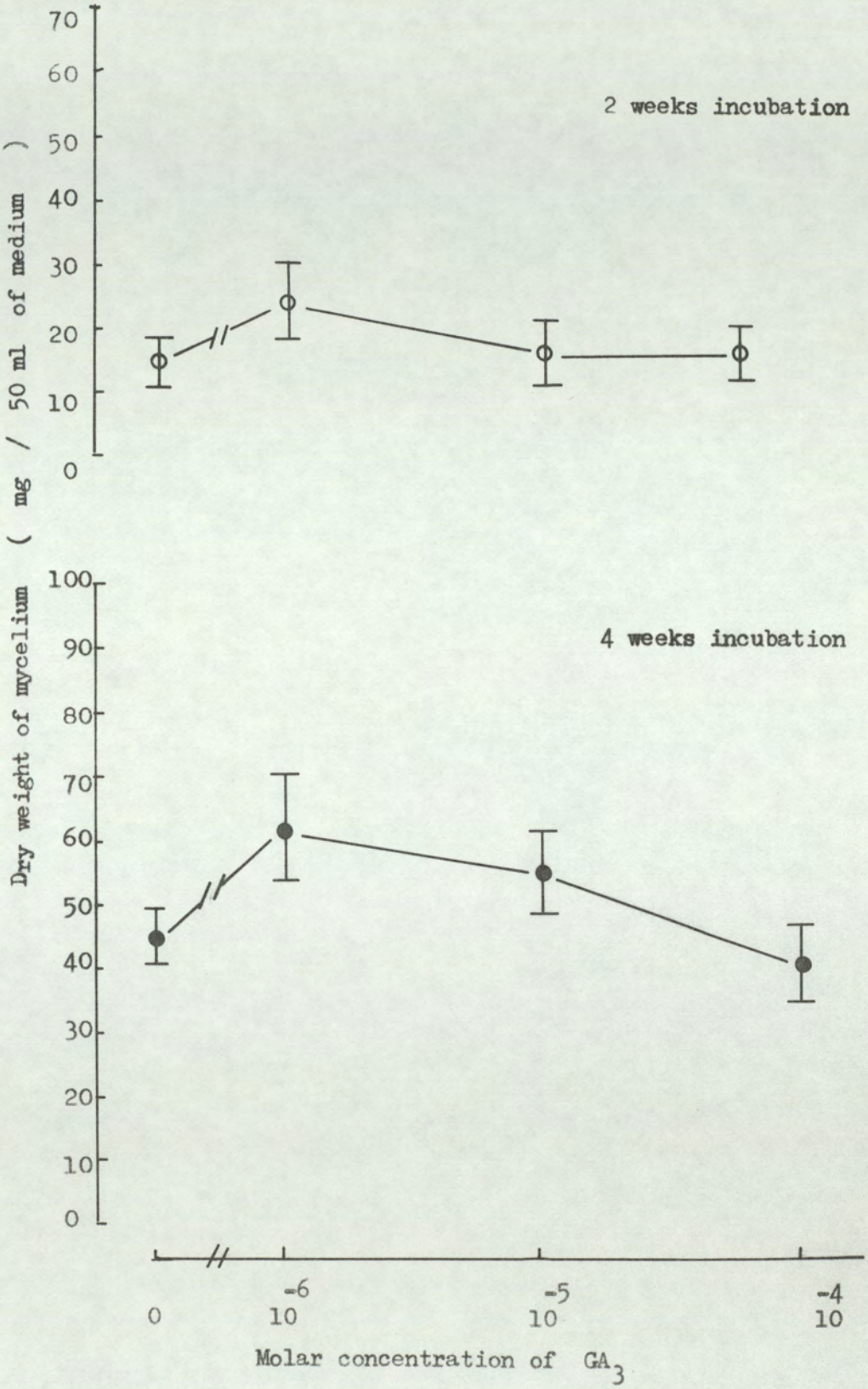
Gibberellic acid (GA_3) increased the dry weight at all test concentrations after two and four weeks of incubation. The only exception was found with the highest concentration used (10^{-4}M), where a slight inhibition occurred.

A significant increase was obtained at concentrations of 10^{-5}M and 10^{-6}M, especially after four weeks of incubation (Figure 3.9, Appendix 1.13). PH decreased slightly at most of tested concentrations during both periods of incubation.

3.2.3 Cytokinin

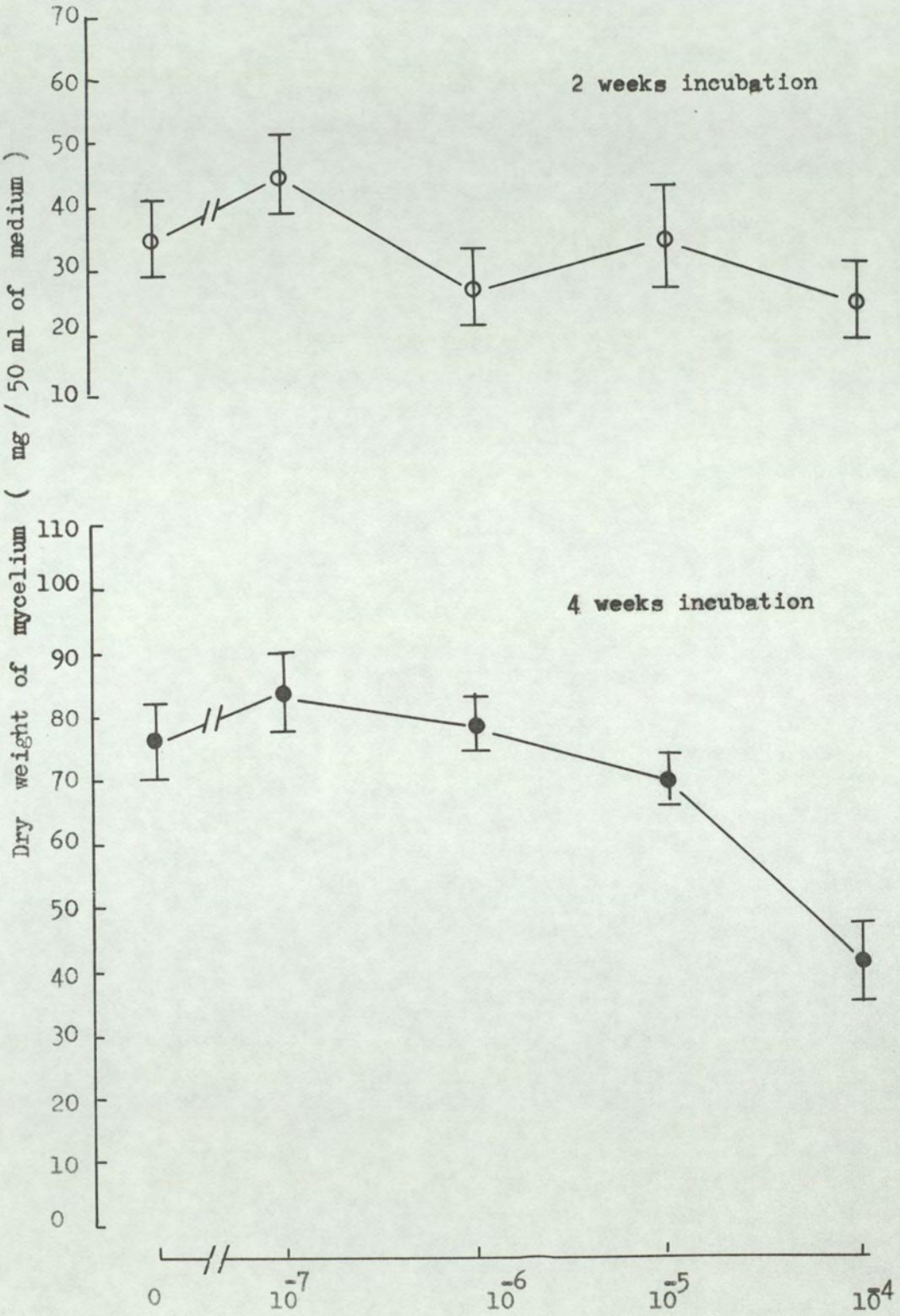
The addition of kinetin at the highest concentration used (10^{-4}M) decreased the dry weight significantly after four weeks of incubation. A significant increase was only obtained at a concentration of 10^{-7}M after both two and four weeks of incubation (Figure 3.10, Appendix 1.14). PH values were generally unaffected at all tested concentrations.

Figure 3.9 :Effect of gibberellic acid on mycelial growth in liquid culture .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.10 : Effect of kinetin on mycelial growth in liquid culture .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

3.2.4 Combination of IAA with GA_3 and K

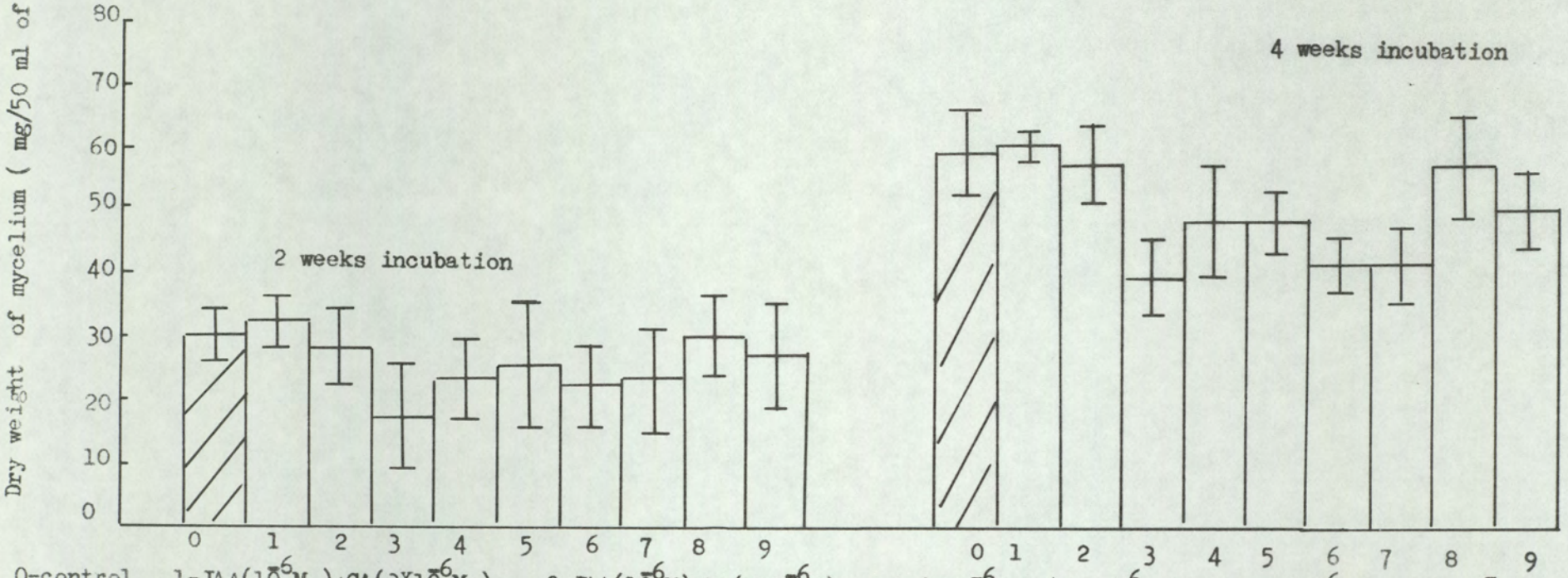
For all treatments, the dry weight of mycelium decreased over the control, except for a combination of 10^{-6}M IAA with 3 X 10^{-6}M GA_3 (Figure 3.11, Appendix 1.15). PH values increased slightly for all tested concentrations.

3.3 Growth on solid substrates

Establishing vegetative growth on a pasteurized compost represents the first stage of artificial techniques of culture employed in commerce. Growth on compost substrates differ from pure culture techniques employed in semi-solid and liquid culture studies, since in pasteurized substrates a wide range of other micro-organisms form part of the total substrate. Thus, their activity in compost supporting vegetative growth may influence growth response to added phytohormones.

The compost was thoroughly mixed with test phytohormone solutions before compacting into glass tubes (15 cm x 4 cm). The same amount of compost mixed with distilled water and compacted in similar tubes, served as a control. The tubes were inoculated with 3-4 grains from a commercial spawn and then incubated at 25°C for ten days, when the measurement of the growth rate and density of the mycelium was undertaken. Five

Figure 3.11 Effect of combination of different phytohormones on mycelial growth in liquid culture .



0=control , 1=IAA(10^{-6}M)+GA($3X10^{-6}$M) , 2=IAA(10^{-6}M)+K ($5X10^{-6}$M) ,3=GA($3X10^{-6}$M)+K($5X10^{-6}$M) , 4=IAA(10^{-6}M)+K($5X10^{-7}$M) , 5=IAA(10^{-6} M) +GA ($3X10^{-7}$M) ,6=K($5X10^{-7}$ M)+GA($3X10^{-7}$M) , 7=IAA(10^{-7}M) +K ($5X10^{-8}$M) ,8=IAA(10^{-7} M) +GA($4X10^{-8}$M) , 9=GA($4X10^{-8}$M) +K($5X10^{-8}$M) .

Note :Vertical bars are the confidence limits of the mean at p=0.05 .

replicates were prepared for each treatment.

For all treatments, the extent of mycelial growth was less than the control. Exceptions were found at a concentration of 3×10^{-6}M of GA_3 and a combination of 10^{-6}M IAA, 3×10^{-6} GA_3 and 5×10^{-6}M K, where significant increases were obtained (Figure 3.12, Appendix 1.16).

Density of the mycelium was increased for all the treatments, but a maximum increase was obtained with kinetin and the combination of IAA, GA_3 and K.

3.4 Conclusions

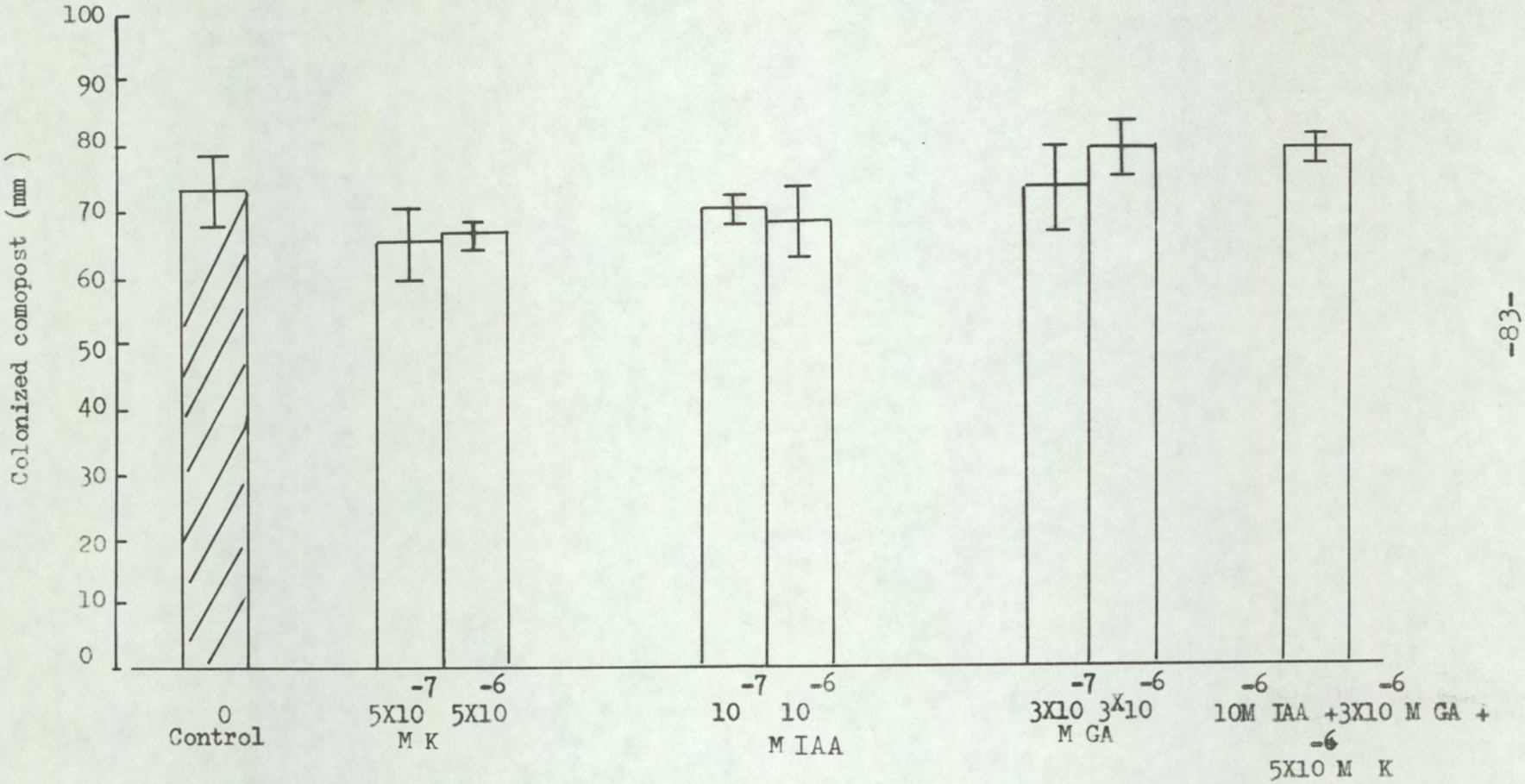
Growth on semi-solid agar

Auxins inhibited the growth at concentrations higher than 10^{-5}M and growth was stimulated at a 10^{-7}M.

Similarly gibberellic acid and kinetin significantly stimulated the growth at 10^{-7}M, but no marked inhibition occurred at the highest concentrations tested (10^{-4}M).

Density and shape of the colony were affected at the highest concentration tested (10^{-3} and 10^{-4}M). Thus, dense and irregular colonies were always associated with the addition of phytohormones at high concentrations.

Figure 3.12 : Effect of phytohormones on mycelial growth on pasteurized compost .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

The effect of combining two or three phytohormones was different from the effect of each phytohormone when used alone. In addition, there was a difference between the effects of combining IAA with K and those of IAA and GA_3. Thus most of the combinations of IAA with K increased the radial growth and sectoring frequency whereas most of those of IAA with GA_3 reduced the radial growth and sectoring frequency. This indicates that phytohormones influence growth not as single entities, but rather by interacting with each other.

The action of phytohormones at the cellular level was demonstrated by addition of IAA which increased cell length at a wide range of concentrations, while GA_3 was very effective in stimulating cell extension at the lowest concentration tested only (4×10^{-8}M). In contrast, kinetin was more effective in reducing cell length at a wide range of concentrations.

Growth on solid substrate

The addition of phytohormones to a pasteurized compost increased the density of the mycelium remarkably. While the optimum concentration for longitudinal growth of the mycelium were 3×10^{-6}M of GA_3 and the combination of 10^{-6}M IAA, 5×10^{-6}M K and 3×10^{-6}M GA_3.

Growth in liquid culture

Phytohormones increased dry weight of the mycelium significantly at a wide range of concentrations including those which caused inhibition of growth on semi-solid agar.

Most of the testedphytohormones were more effective in stimulating growth in liquid culture than on semi-solid agar. For example, 10^{-7}M of IAA and 10^{-6}M of GA_3 increased the growth by 9.8 and 12.9% on semi-solid agar while the same concentrations increased the dry weight in liquid culture by 108 and 37%, respectively.

PH values were generally unaffected by adding phytohormones, except for a general tendancy for an increase of pH in culture media which supported greater amounts of mycelial growth.

PART II PHYTOHORMONES AND REPRODUCTIVE GROWTH

3.5 Growth on solid substrate

The important transition from vegetative to reproductive growth in A.bisporus is a result of a complex range of factors and the mechanisms are not fully understood. For fruit bodies to be formed, a layer of soil, termed a casing soil, is placed over the surface of compost colonized by mycelium. Cultures are aerated to maintain low levels of carbon dioxide and the temperature is reduced from an optimum of 25°C to an optimum of 16°C.

The formation of fruit bodies is also associated with bacteria in the casing soil.

In order to study the effects of phytohormones on the formation, form and yield of fruit bodies it is necessary to use conventional methods of culture using a pasteurized compost and a peat chalk casing soil.

To determine the effects of phytohormones when applied to both compost and casing soil substrates, 60 ml of a given phytohormone solution was added to the surface of both layers at five days before casing application, five days following casing, immediately following the first harvest (flush or break) and following second harvest (see section 2.4.4).

The following parameters were studied according to methods outlined in sections 2.4.5 and 2.7.1:

a - Shape of sporophore

b - Total yield of harvested sporophores

c - Dry weight of sporphore

d - Stipe length and pileus diameter

e - Average weight of individual sporophore

f - Protein content

g - Primordium and sporophore number

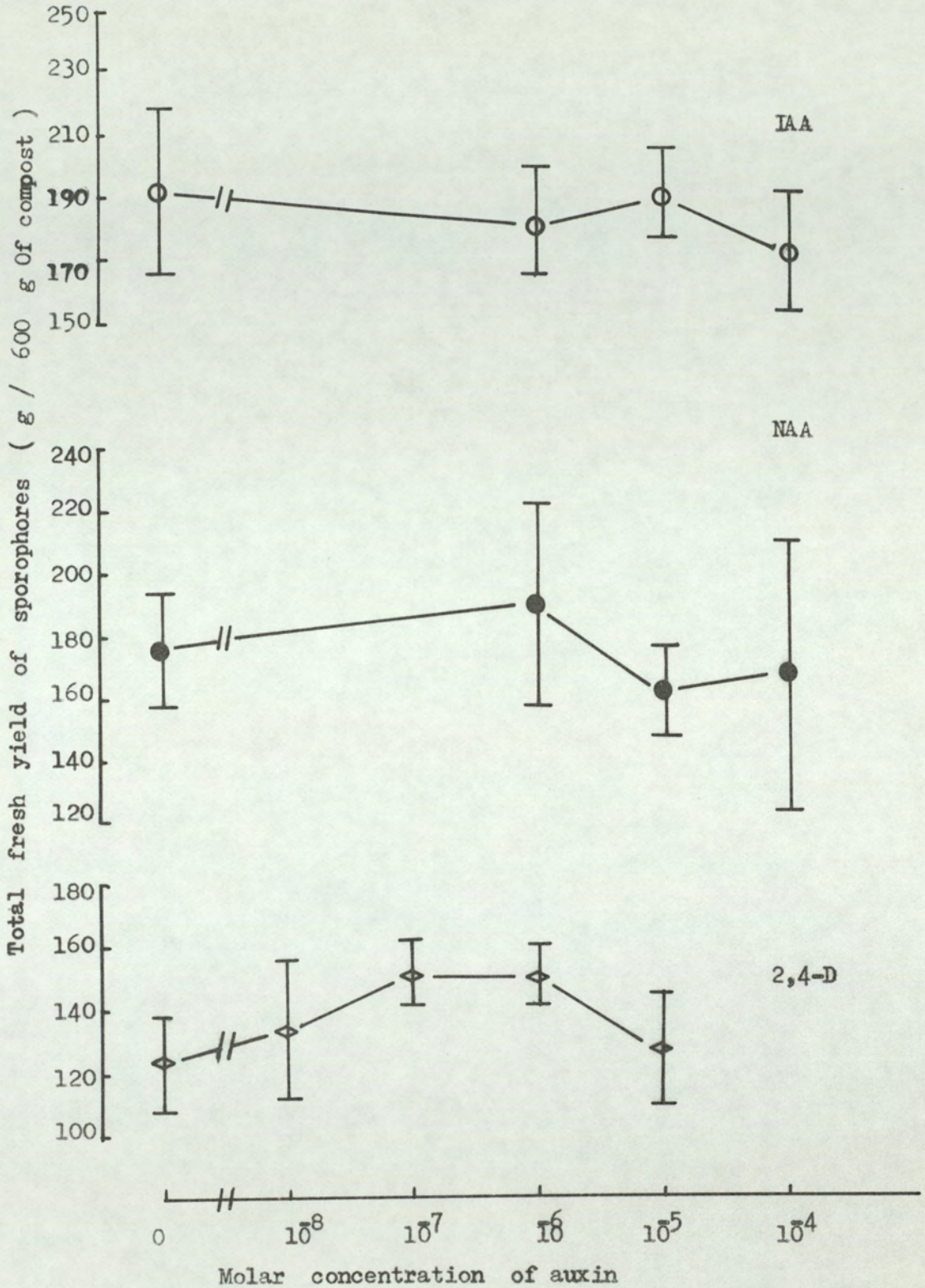
3.5.1 Auxins

IAA had an inhibitory effect on the total yield of harvested sporophores, especially at the highest concentration used (10^{-4}M) (see Figure 3.13). Similarly, NAA reduced the total yield, especially at concentrations of 10^{-4}M and 10^{-5}M, but a slight increase (7.9%) was obtained at the lowest concentration tested 10^{-6}M (Figure 3.13, Appendix 2.2).

In contrast, 2,4-D promoted an increase in the total yield over a wide range of concentrations. A significant increase (at $p = 0.05$) was obtained at concentrations of 10^{-7} and 10^{-6}M (Figure 3.13, Appendix 2.3).

In general, the tested auxins reduced slightly the dry weight of sporophore, especially at the highest

Figure 3.13 :Effect of Auxins on total yield of sporophores.



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

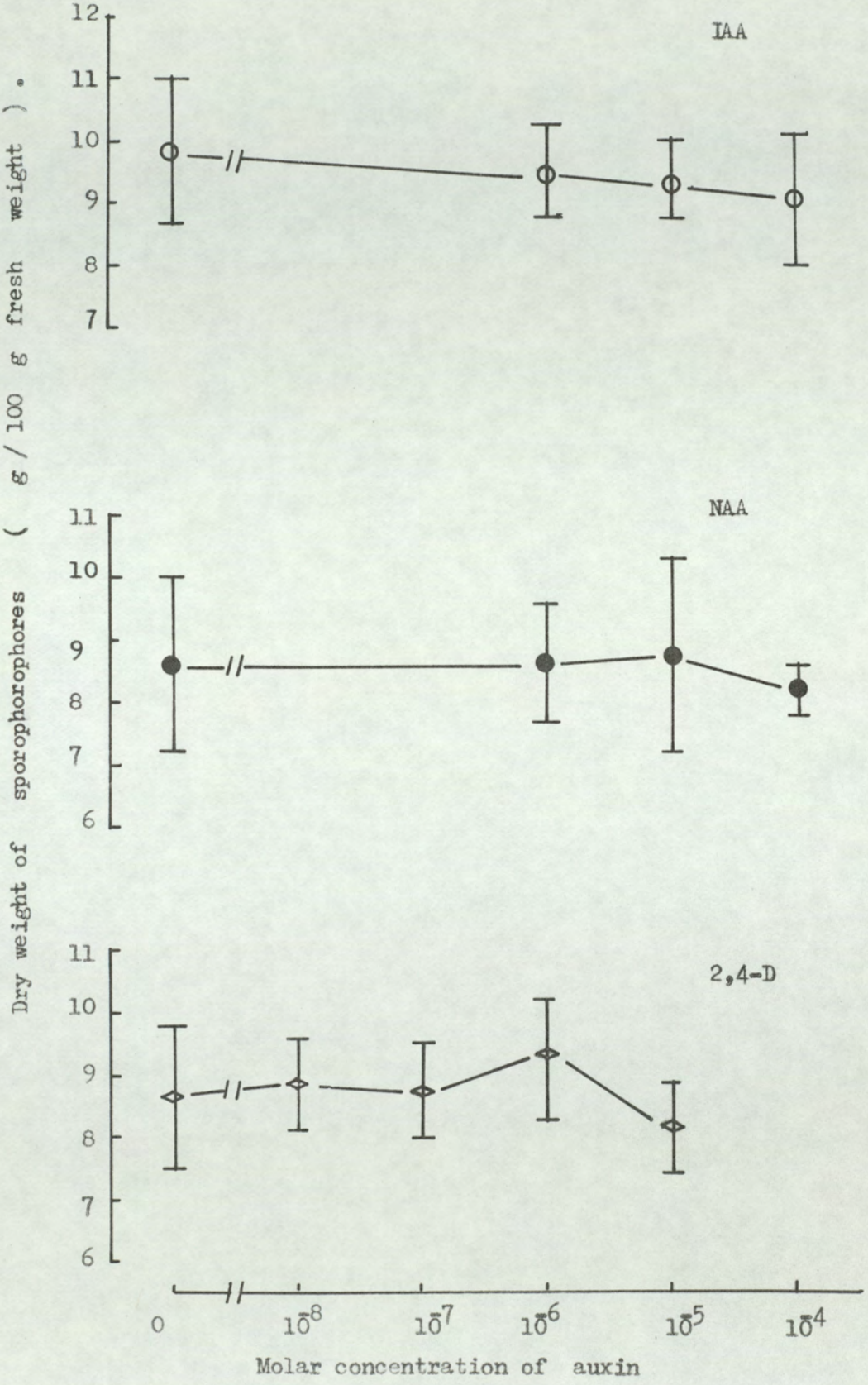
concentration applied (see Figure 3.14 and Appendices 2.1, 2.2, 2.3).

The addition of auxin also influenced the initiation of primordia and development of sporophores. Thus primordium numbers were significantly reduced, especially at the highest concentration applied (see Figure 3.15), whereas the sporophore number was slightly reduced over a wide range of concentration of IAA and NAA, but it was a significant at concentrations of 10^{-4} and 10^{-5}M of NAA. In contrast, 2,4-D significantly increased the sporophore number at concentrations of 10^{-5} and 10^{-6}M (Figure 3.16, Appendix 2.3).

Stipe length was also influenced by adding auxins. All tested auxins increased the stipe length at concentrations of 10^{-5} to 10^{-6}M. But IAA was the most effective auxin in increasing the stipe length. Thus, it significantly increased the stipe length at 10^{-6}M ($p = 0.05$) (see Figure 3.17).

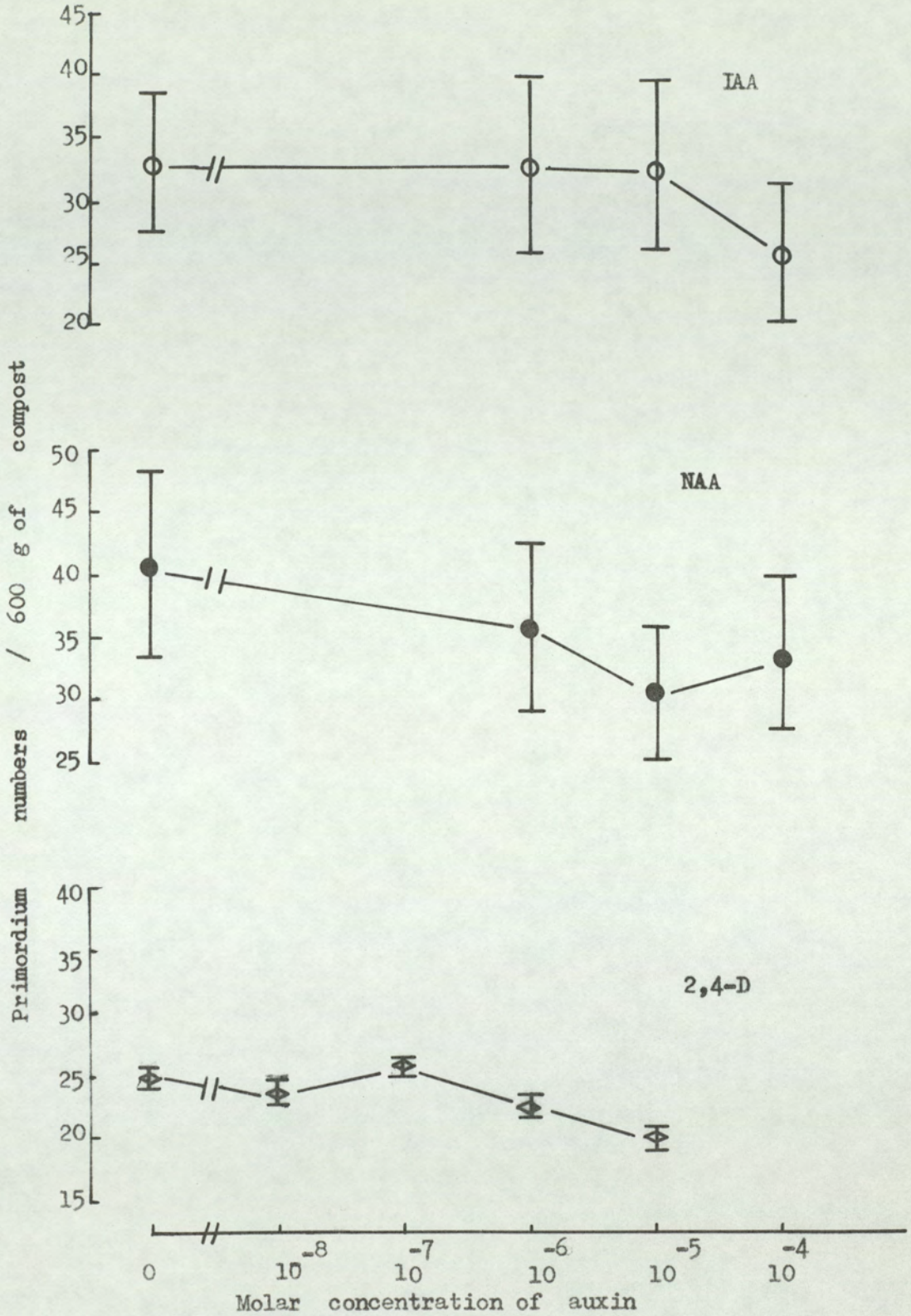
The addition of auxins also influenced pileus diameters. Thus, IAA increased it slightly at concentrations of 10^{-6} and 10^{-5}M, while there was almost a consistent relationship between pileus diameter and NAA concentration applied. A significant increase was obtained at 10^{-4} and 10^{-5}M of NAA. While 2,4-D

Figure 3.14 :Effect of auxins on dry weight of sporophores .



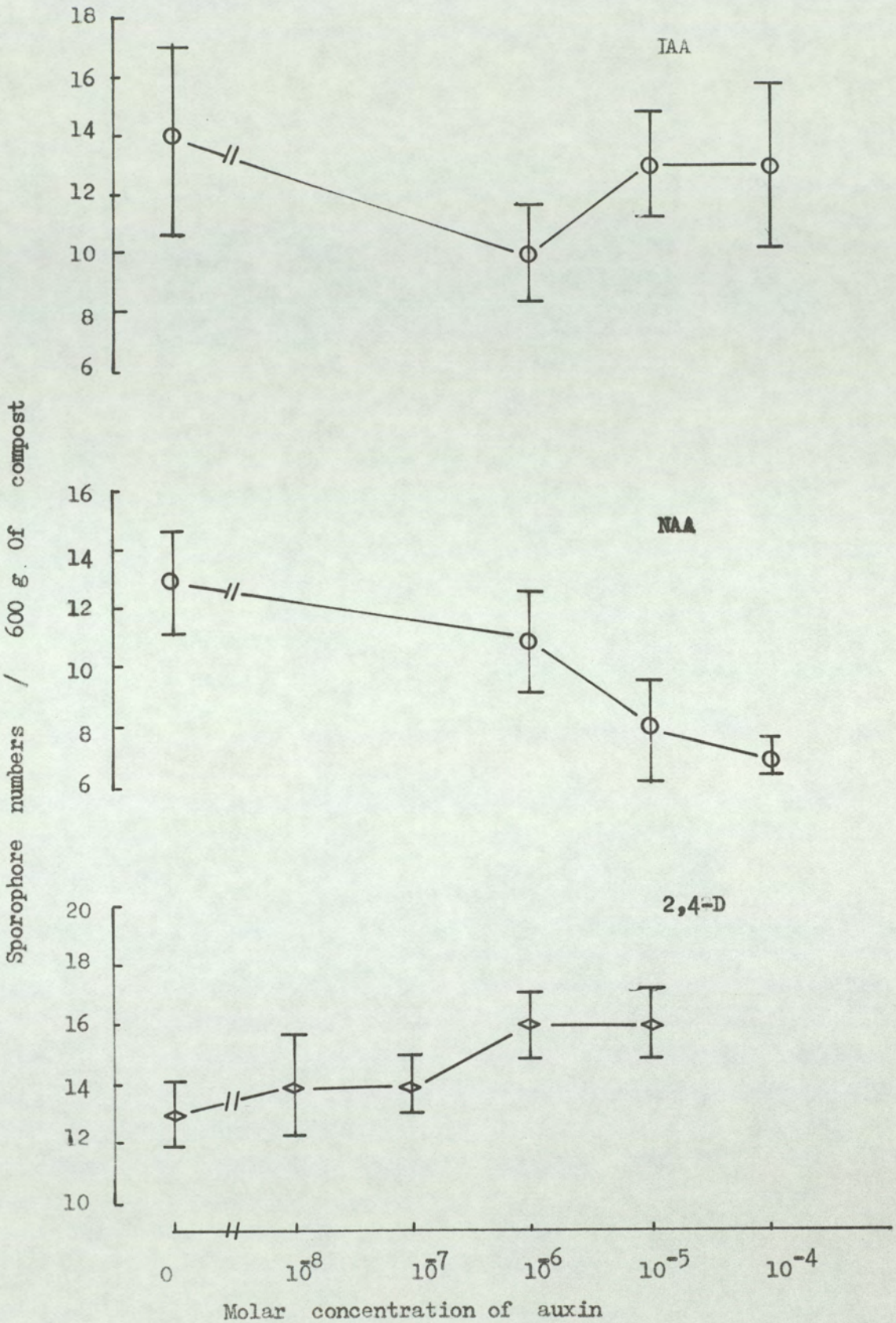
Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.15 :Effect of auxins on primordium numbers .



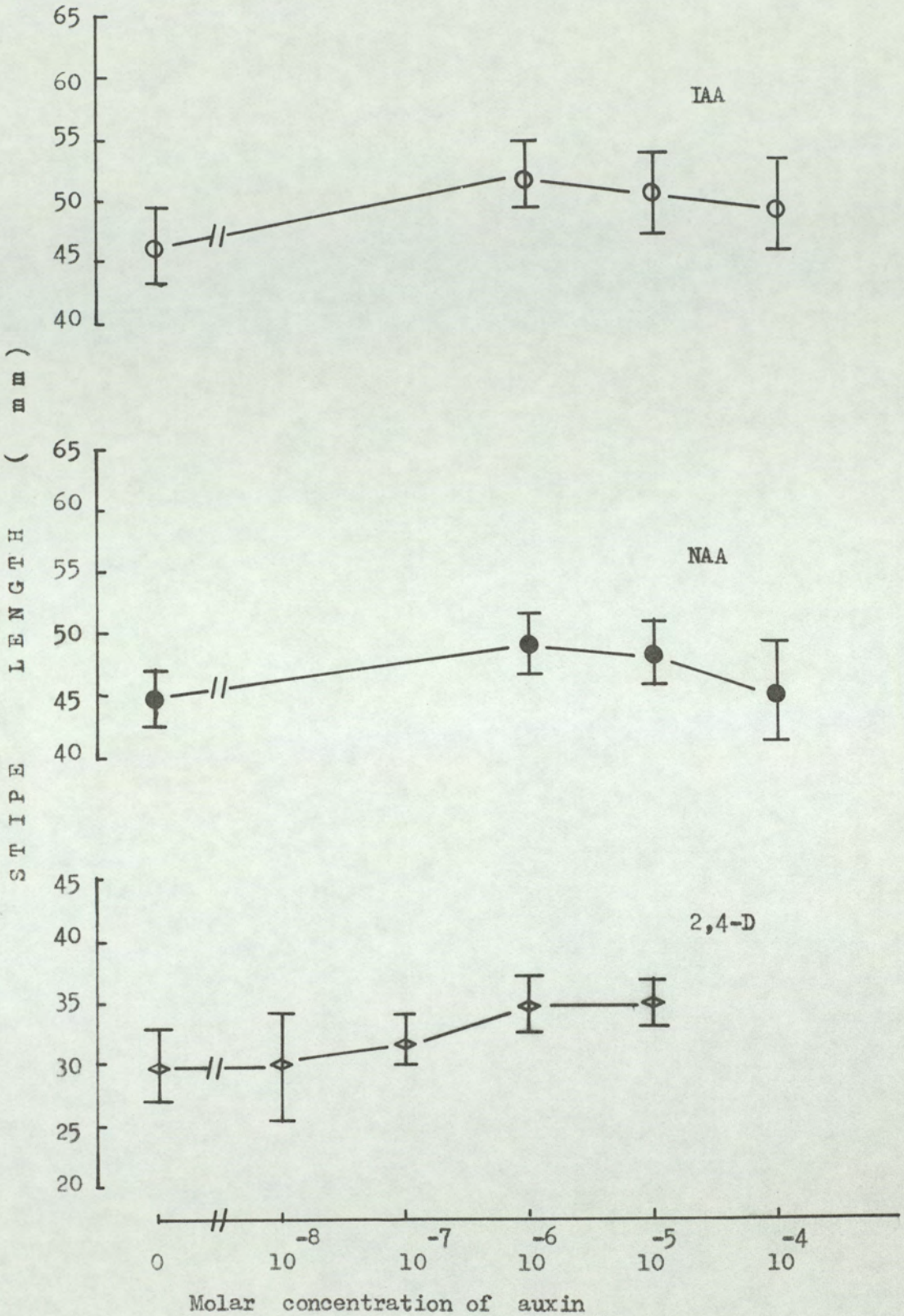
Note :Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.16 :Effect of auxins on sporophore numbers .



Note :Vertical bars are the confidence limits of the mean at p= 0.05 .

Figure 3.17 :Effect of auxins on stipe length .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

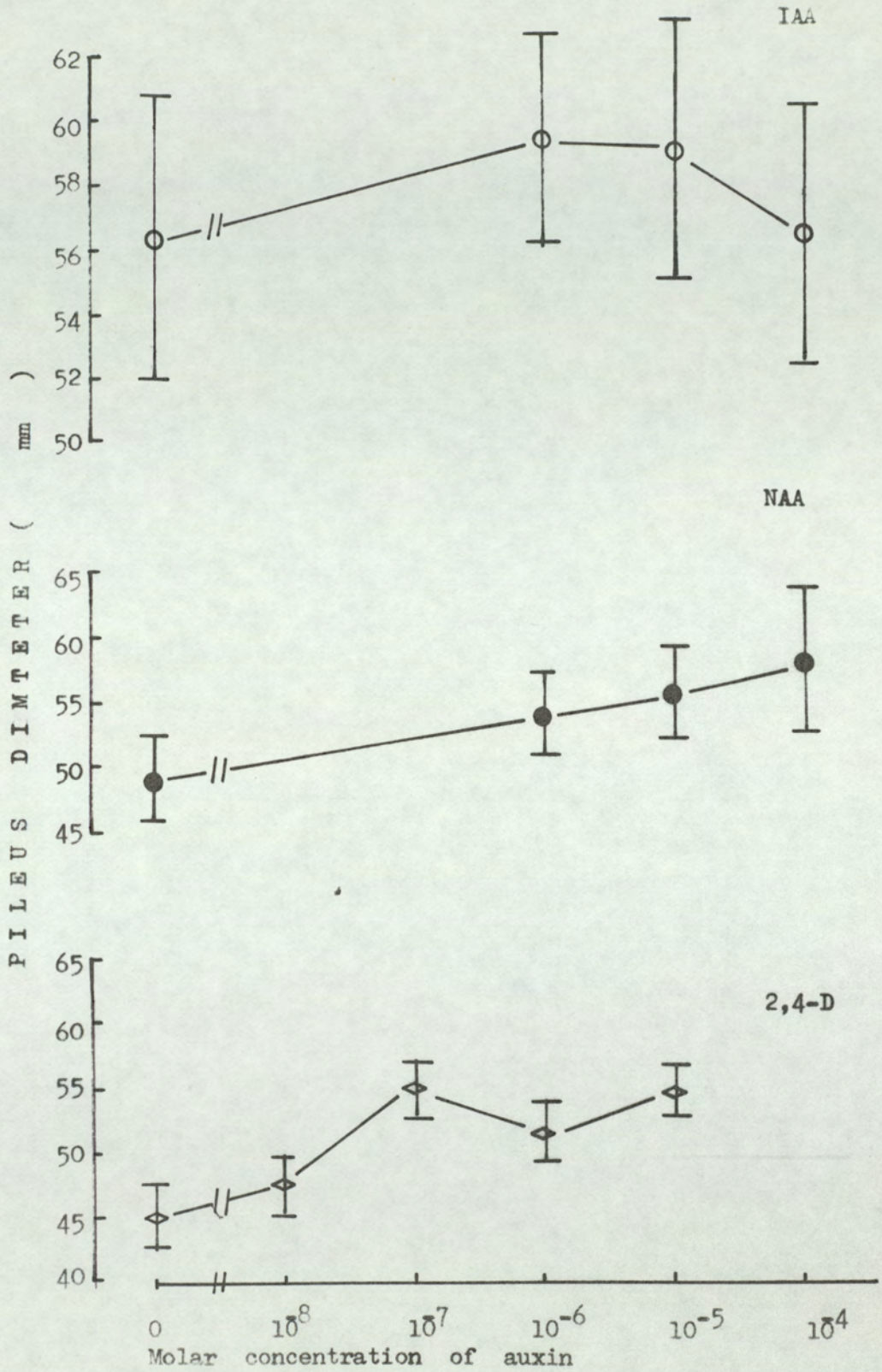
significantly increased pileus diameter at a wide range of concentration viz 10^{-5}, 10^{-6} and 10^{-7}M (Figure 3.18).

Although the size and the average weight of sporophore were affected by addition of auxins, the common shape remained very similar to that of the control. The most effective auxin in increase the average weight of sporophore was NAA (Table 2.1).

Table 2.1 Effect of auxins on the average weight of sporophore

Auxins	concentration applied (mole)	average weight of sporophore (g)
IAA	0	13.78
	10^{-4}	13.3
	10^{-5}	14.76
	10^{-6}	18.5
NAA	0	13.5
	10^{-4}	25.5
	10^{-5}	19.47
	10^{-6}	16.8
2,4-D	0	9.5
	10^{-8}	9.5
	10^{-7}	10.9
	10^{-6}	9.5
	10^{-5}	8

Figure 3.18 : Effect of auxins on pileus diameter.



Note : Vertical bars are the confidence limits of the mean at p=0.05 .

Protein content was also influenced by addition of auxins. The most effective auxin in increasing protein content was 2,4-D. While IAA and NAA slightly increased it at a concentration of 10^{-6}M (see Figure 3.19).

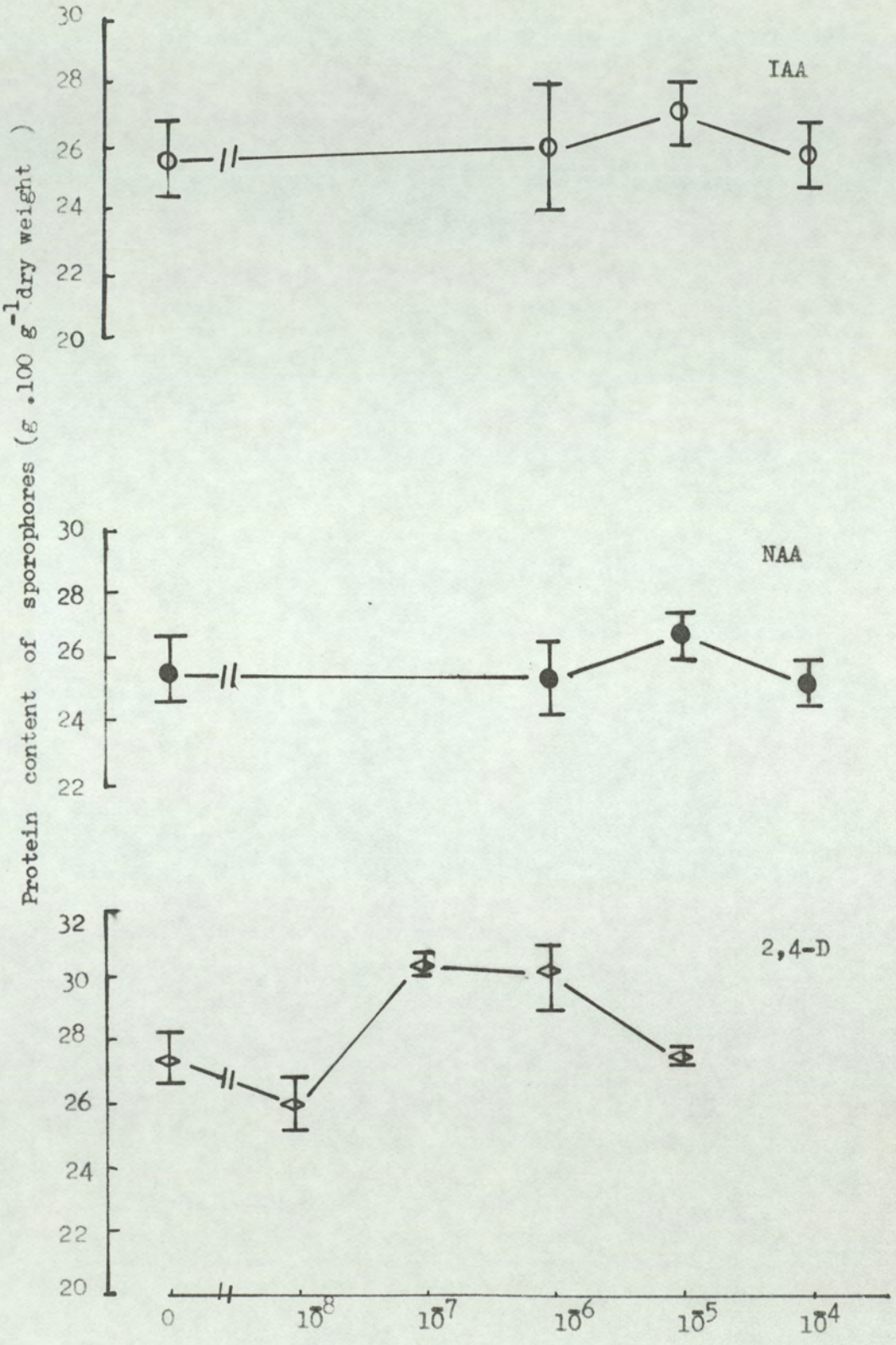
3.5.2 Gibberellin

The addition of gibberellic acid slightly increased the total yield of harvested sporophores at a concentration of 10^{-6}M only while it reduced the yield at concentrations higher than 10^{-6}M (Figure 3.20, Appendix 2.4). Also gibberellic acid slightly reduced the dry weight of sporophores and there was a significant reduction at the highest concentration tested 10^{-4}M (Figure 3.21).

Stipe length was generally unaffected by adding GA_3 except for a concentration of 10^{-5}M, where a significant decrease took place. While pileus diameters were slightly decreased at all tested concentrations (Figure 3.22).

For all treatments, sporophore numbers decreased considerably, in contrast the primordium numbers increased slightly over a wide range of tested concentrations, except at a concentration of 10^{-4}M, where a slight reduction occurred (Figure 3.23).

Figure 3.19 : Effect of auxins on protein content of sporophores .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3. 20 : Effect of gibberellic acid on total yield.

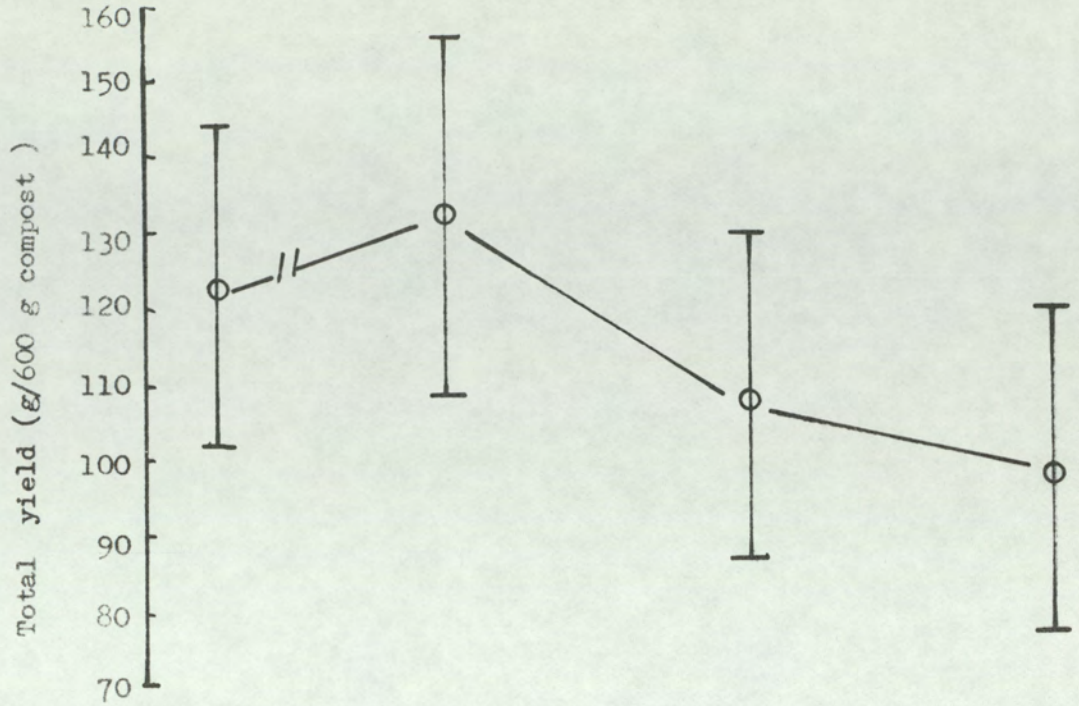
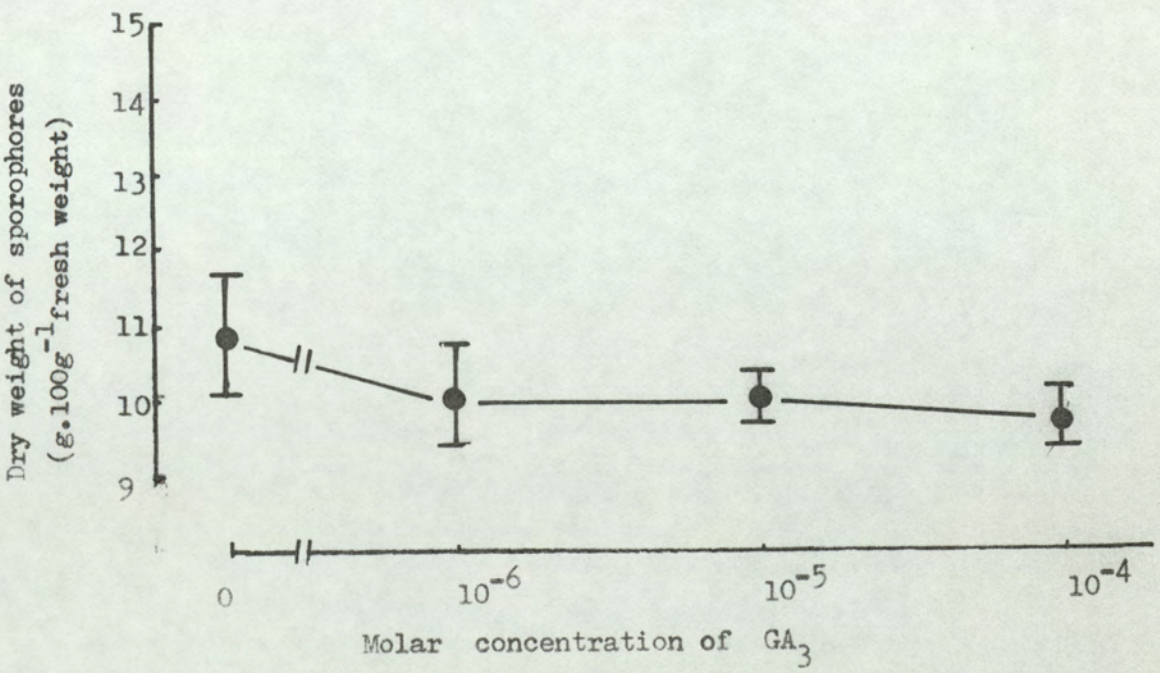


Figure 3.21 :Effect of GA_3 on dry weight of sporophores.



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.22 :Effect of GA_3 on stipe length and pileus diameter.

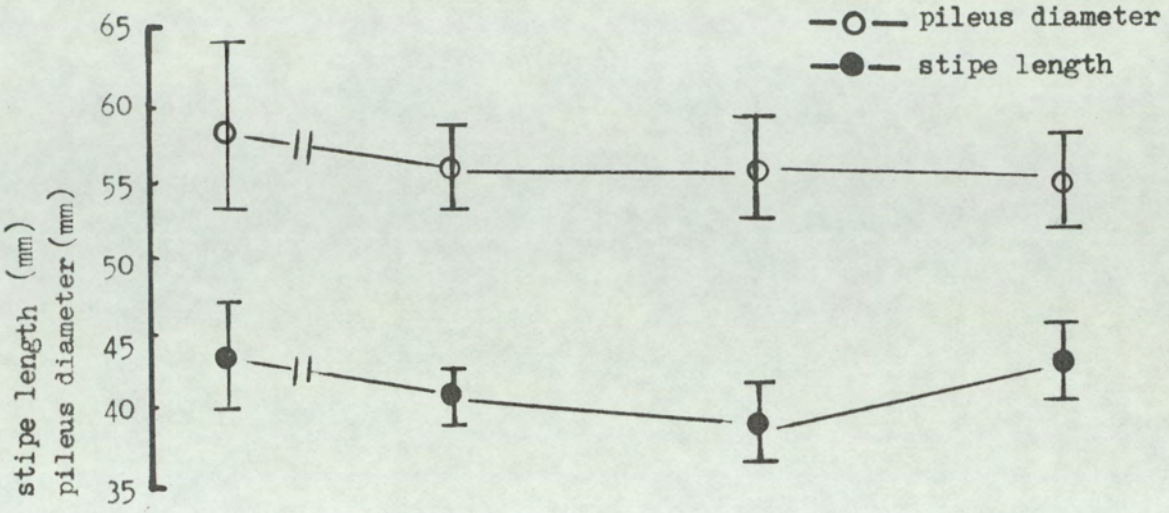
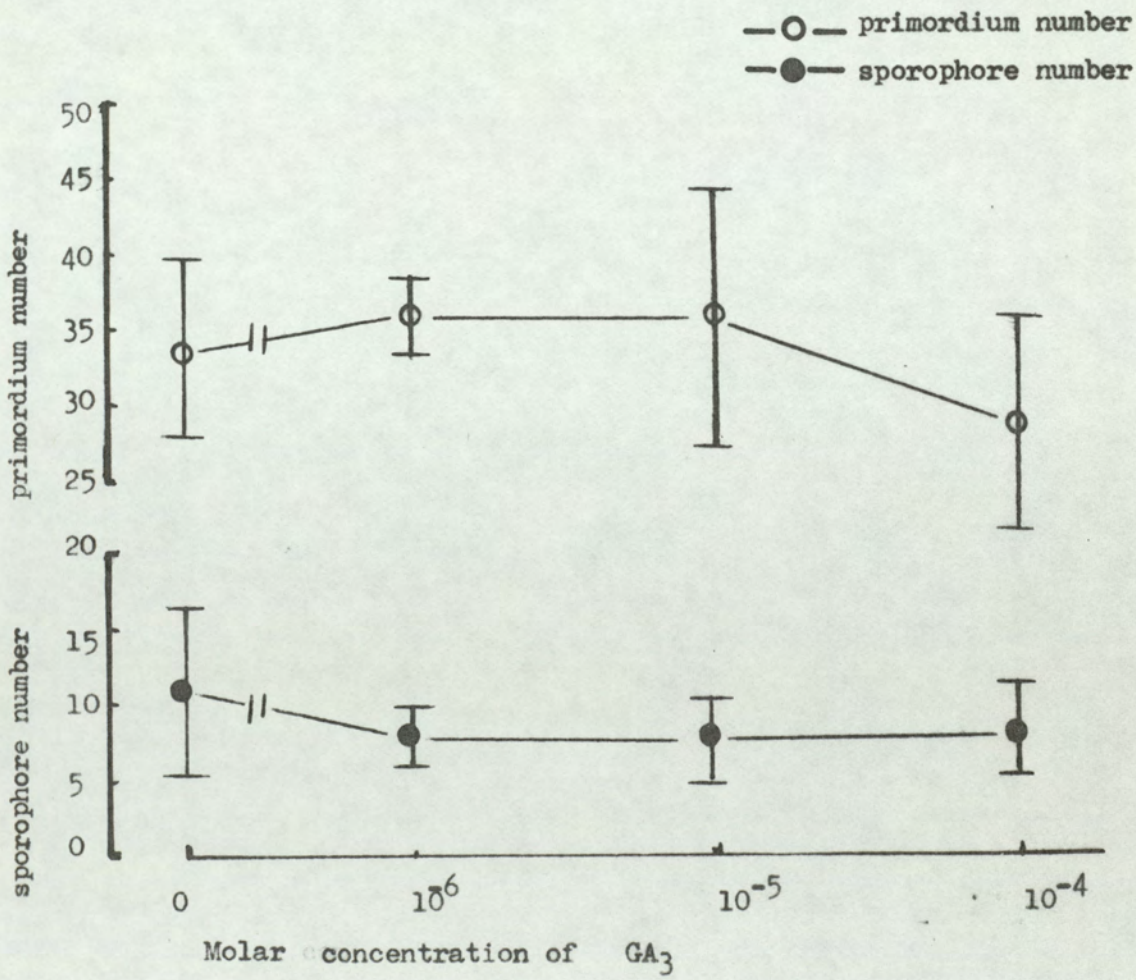


Figure 3.23 :Effect of GA_3 on primordium and sporophore number.



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Although significant increase in the protein content was obtained at concentrations of 10^{-4} and 10^{-6}M, there was no consistent correlation between the protein content and GA_3 concentration applied (Figure 3.24).

In general, the shape of sporophore was unaffected by addition of GA_3 but the average weight of individual sporophore increased considerably at the lowest concentration tested 10^{-6}M (Table 2.2).

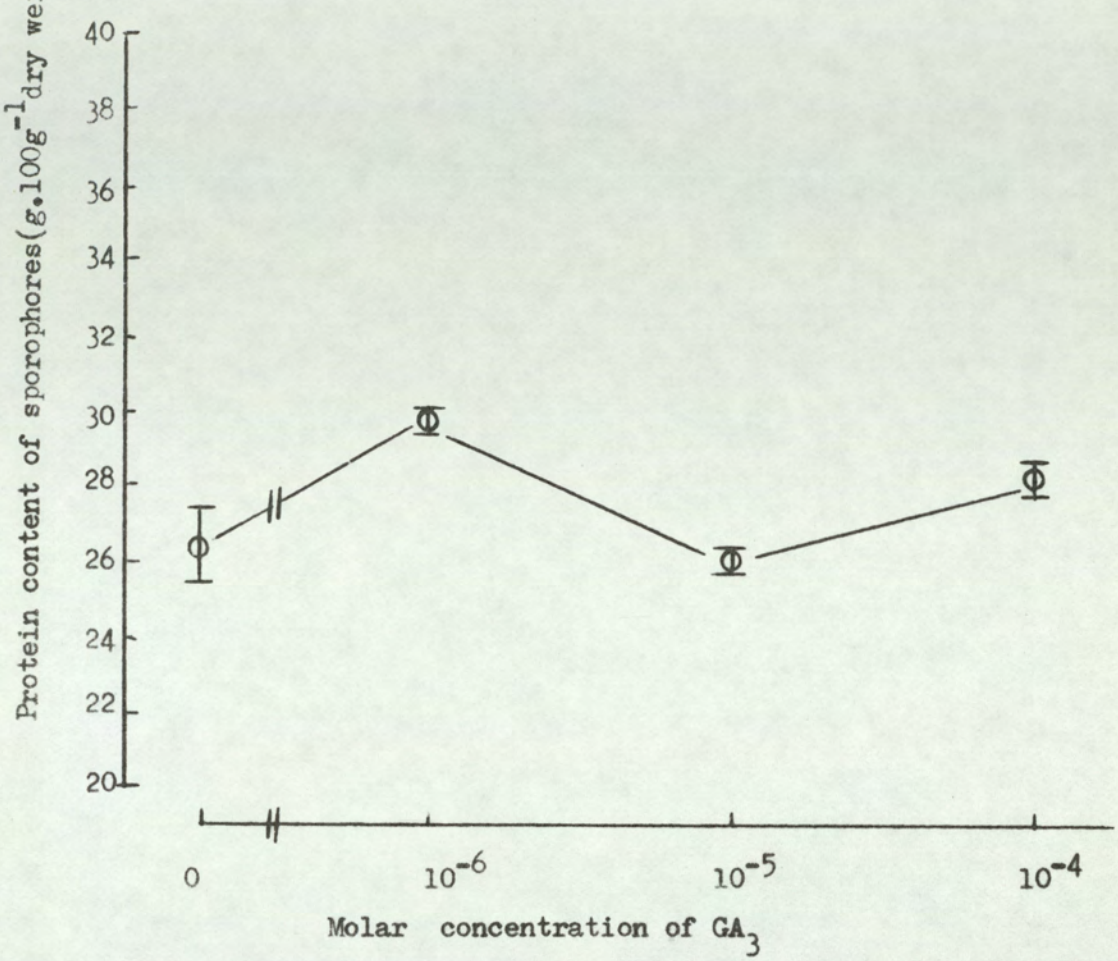
Table 2.2 Effect of gibberellic acid on the average weight of sporophore

Concentration applied (mole)	Average weight of sporophore (g)
0	11.1
10^{-4}	12.39
10^{-5}	13.55
10^{-6}	16.5

3.5.3 Cytokinin

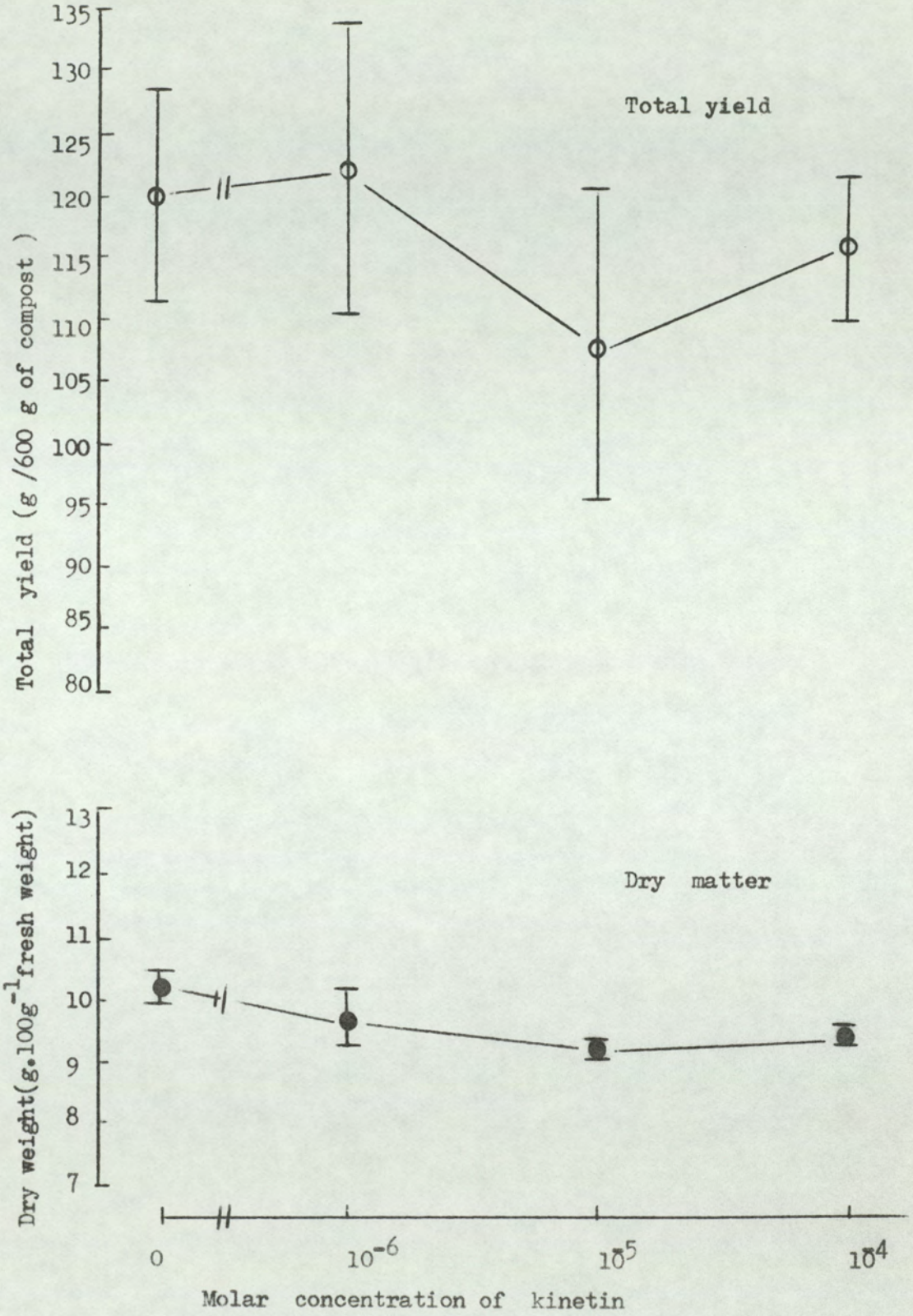
For all treatments, the total yield of harvested sporophores was unaffected, except at a concentration of 10^{-5}M, which caused a slight reduction in yield. While the dry weight of sporophores were significantly reduced at all concentrations (Figure 3.25, Appendix 2.5).

Figure 3. 24 :Effect of GA_3 on protein content of sporphores .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3. 25:Effect of kinetin on total yield and dry weight of sporophores .



Note :Vertical bars are the confidence limits of the mean at p=0.05 .

Stipe length and pileus diameter were also affected by adding kinetin. Thus there was an almost consistent relationship between pileus diameter and kinetin concentration applied, whereas the stipe length increased significantly at concentrations of 10^{-6}M and 10^{-4}M (see Figure 3.26).

The sporophore number was affected by addition of kinetin, thus a significant reduction occurred at a concentration of 10^{-5}M. Whereas the primordium number was significantly increased at a concentration of 10^{-6}M. It was significantly reduced at a 10^{-5}M (Figure 3.27).

Although the addition of kinetin increased the total protein content significantly, there was no consistent relationship between the protein content and kinetin concentration used (Figure 3.28).

As with auxin and gibberellic acid (GA_3) kinetin had no remarkable effect on the common shape of the sporophore, except for increasing the average weight of the sporophore at all tested concentrations (Table 2.3).

Table 2.3 Effect of kinetin on the average weight of sporophore.

Concentration applied (mole)	Average weight of sprophore (g)
0	24
10^{-6}	30.5
10^{-5}	36
10^{-4}	29

Figure 3.26 :Effect of kinetin on pileus diameter and stipe length .

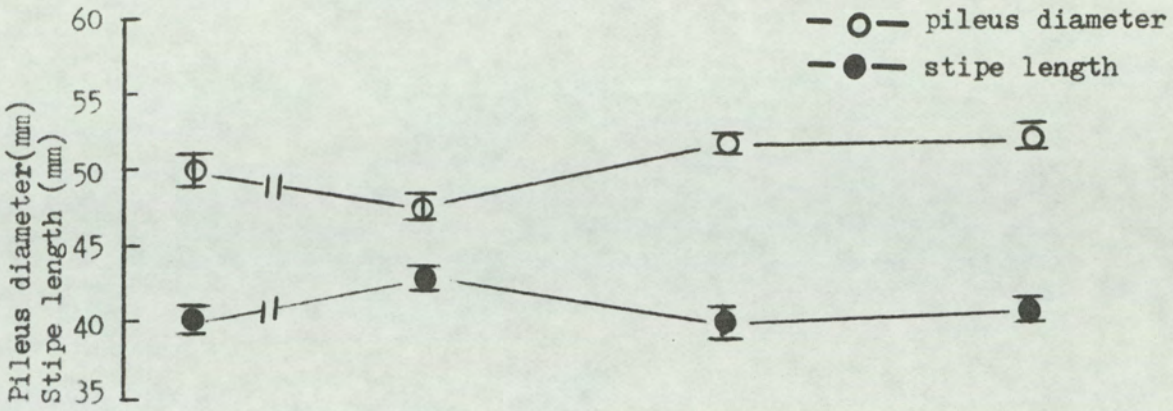
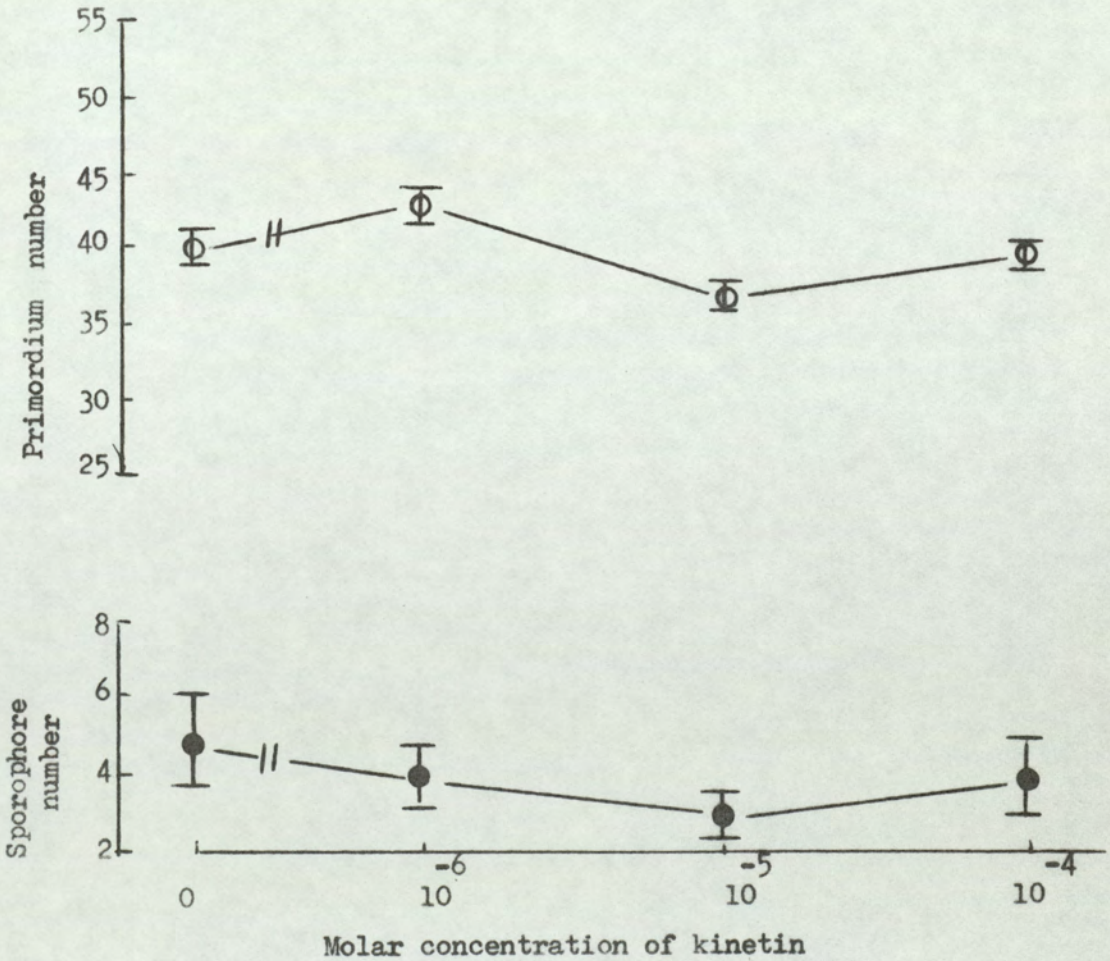
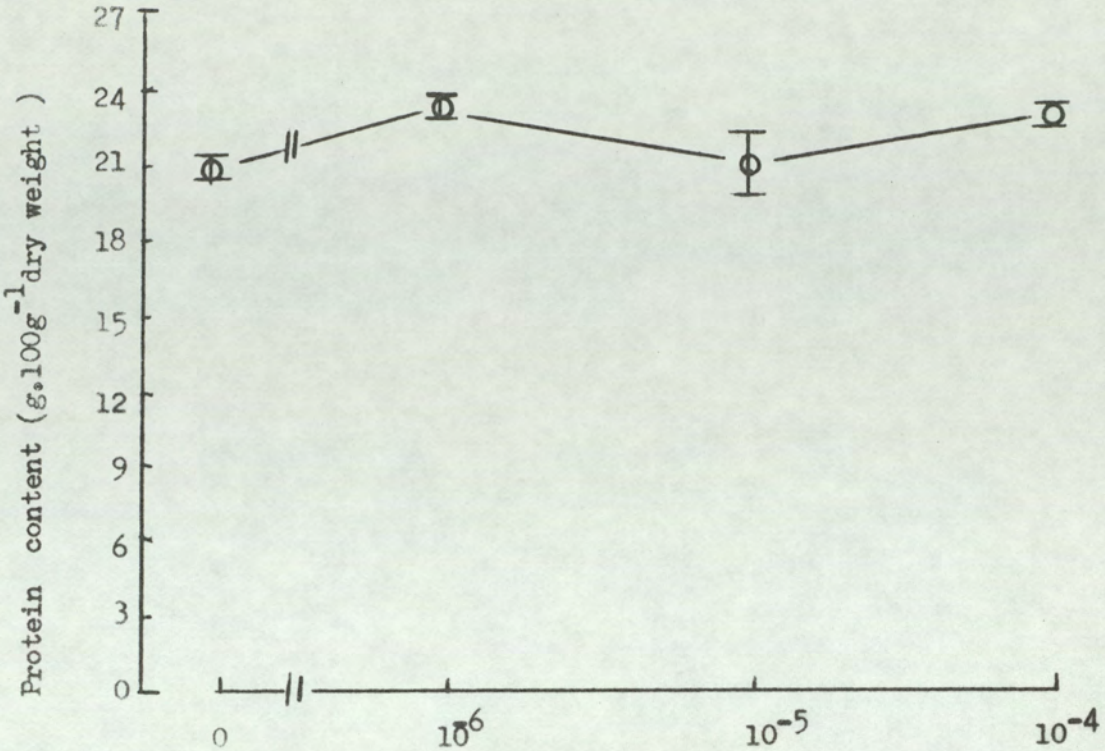


Figure 3.27 :Effect of kinetin on primordium and sporophore number .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.28 :Effect of kinetin on protein content of sporophores .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

3.5.4 Combination of phytohormones

The combination of three phytohormones, IAA, GA_3 and K, reduced the total yield but not significantly. The results were plotted in Figure 3.29 and tabulated in Appendix 2.6.

Dry matter of sporophore was significantly decreased with a combination of the three hormones at a concentration of 10^{-5}M (see Figure 3.29).

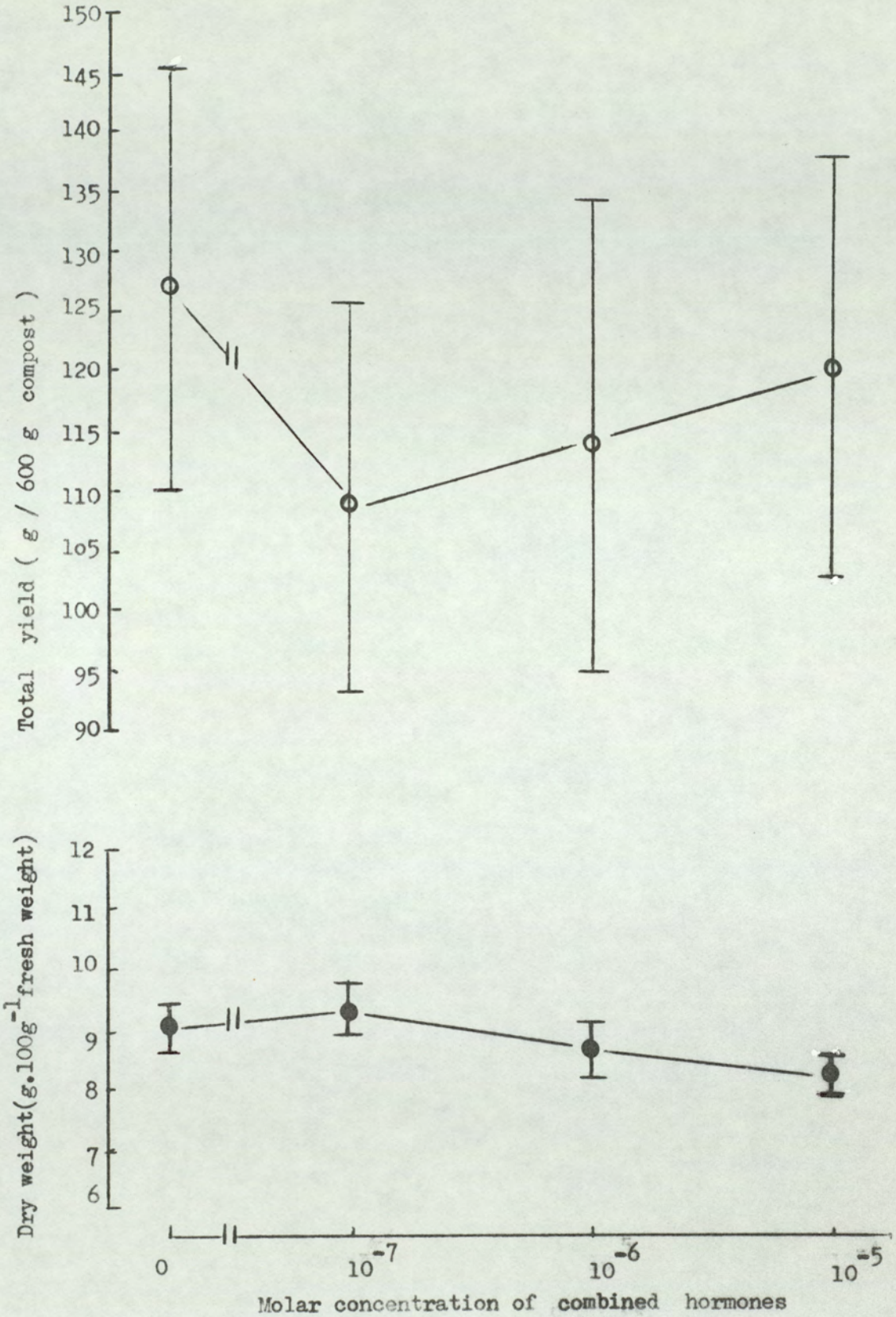
For all combinations the primordium number was unaffected, except for a combination at a concentration of 10^{-7}M only, where a significant increase was obtained. In contrast, sporophore number decreased for all test combinations, but the significant reduction occurred at 10^{-7}M and 10^{-6}M (see Figure 3.30).

Pileus diameter and stipe length increased significantly with a combination of 10^{-5}M, but pileus diameter decreased significantly with combination of 10^{-6}M (see Figure 3.31).

Also a significant increase in protein content took place with combination of 10^{-7}M only (Figure 3.32).

The shape of sporophore was normally unaffected and also the average weight of the sporophore was not markedly affected (Table 2.4).

Figure 3. 29:Effect of combination of IAA, GA_3 and K on total yield and dry weight of sporophores .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.30 :Effect of combination of IAA, GA_3 and K on primordium and sporophore numbers .

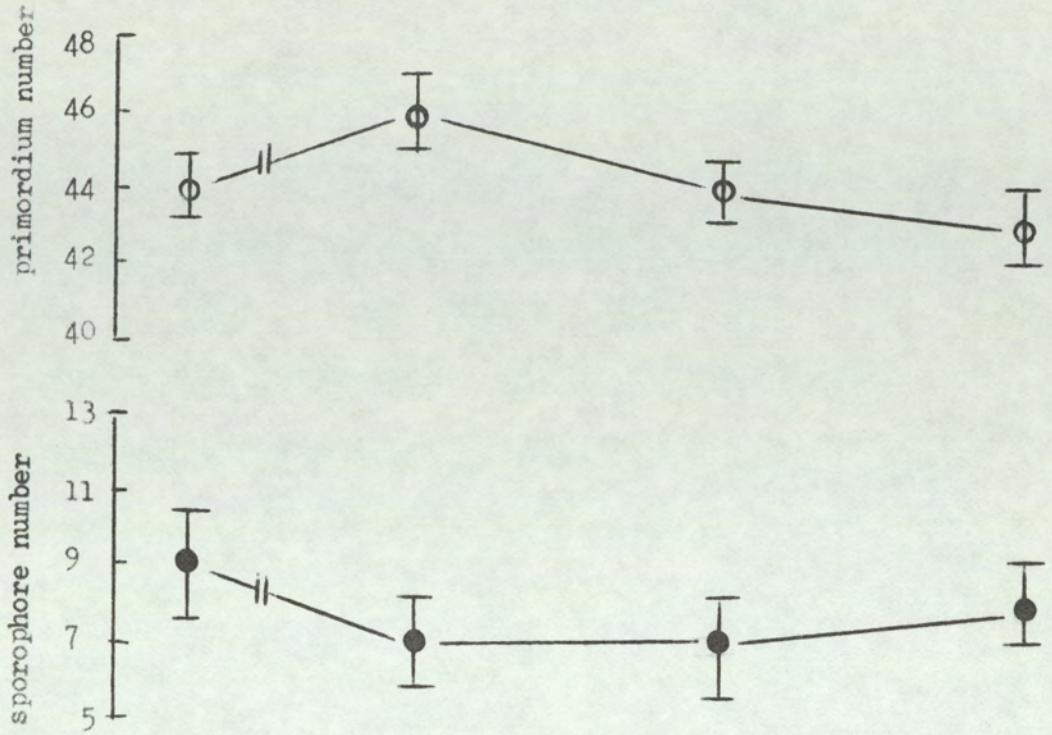
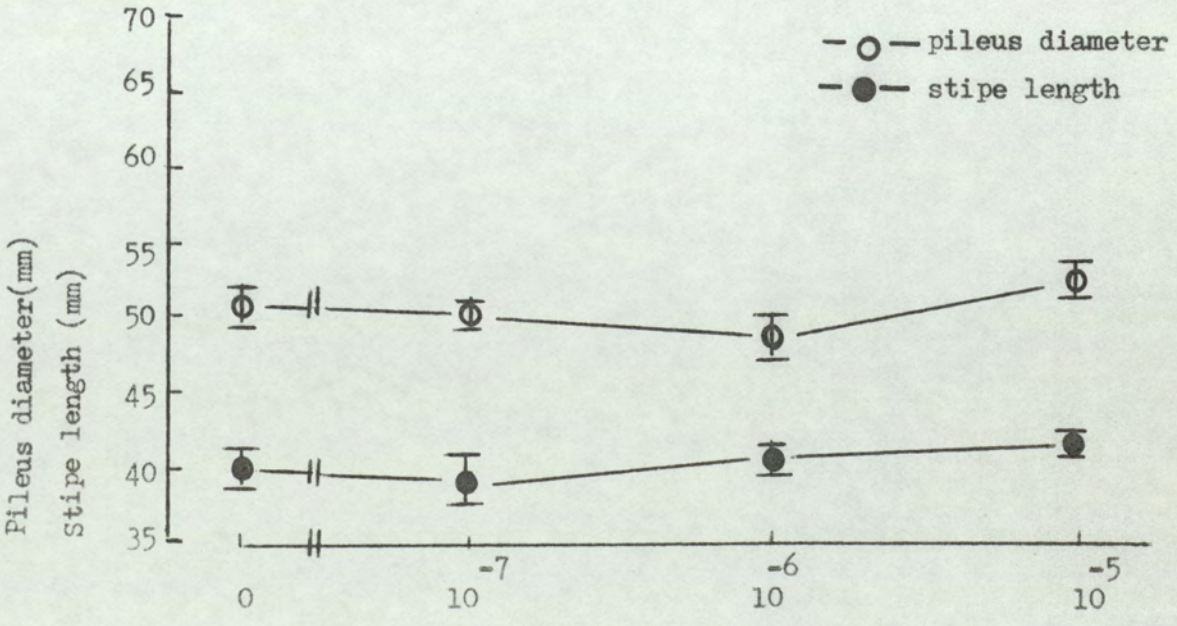
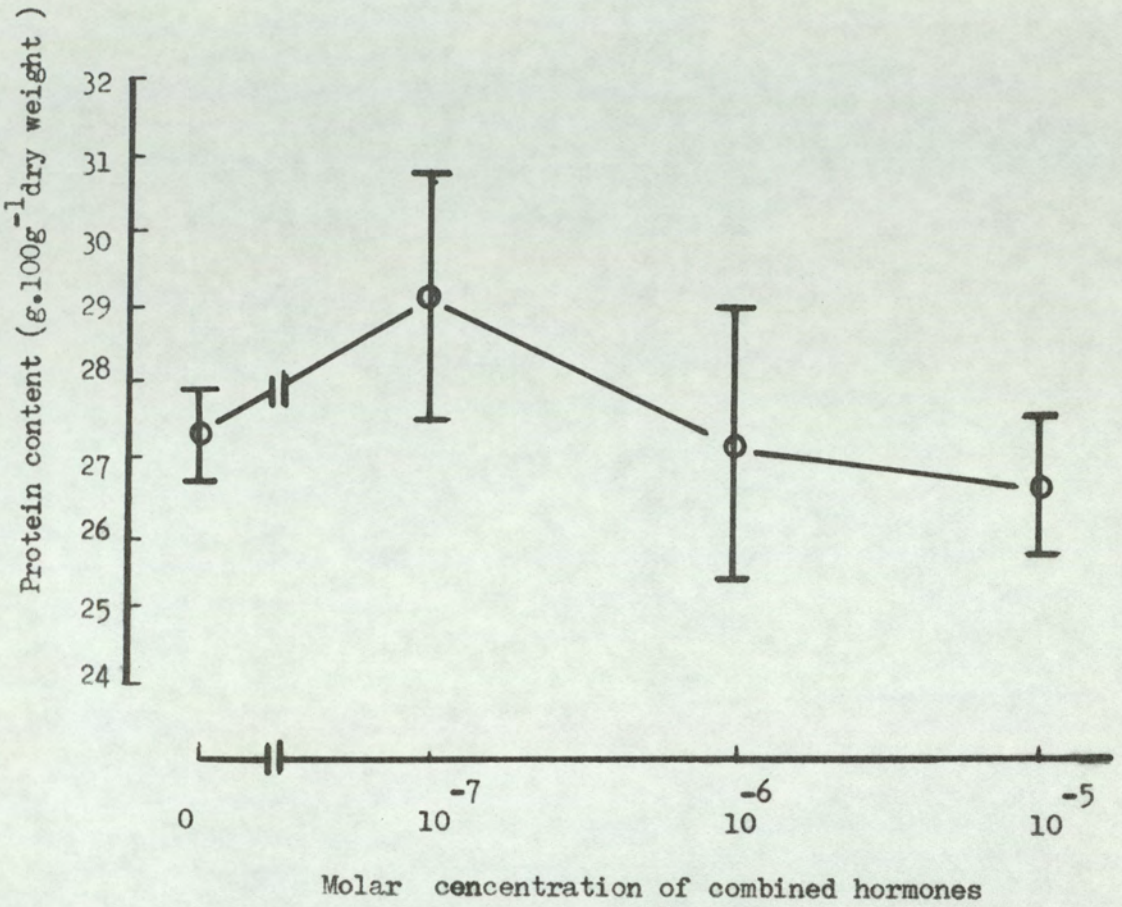


Figure 3.31 :Effect of combination of IAA, GA_3 and K on pileus diameter and stipe length .



Note:Vertical bars are the confidence limits of the mean at p=0.05 .

Figure 3.32 :Effect of combination of IAA, GA_3 and K on protein content of sporophores .



Note:Vertical bars are the confidence limits of the mean at $p = 0.05$.

Table 2.4 Effect of combining three phytohormones on the average weight of individual sporophore

Concentration applied (mole)		Average weight of sporophore (g)
	0	14.19
Combination of	10^{-7}	15.5
Combination of	10^{-6}	16.4
Combination of	10^{-5}	15.0

3.5.5 Conclusions

Auxins considerably reduced the total yield of harvested sporophores at the highest concentration used, 10^{-4}M, except 2,4-D which increased the yield significantly at a wide range of concentrations. Yield was increased by 7.9 and 23% by adding a 10^{-6}M of NAA and 2,4-D, respectively.

It seems that2,4-D is the most effective auxin in stimulating sporophore development, thus it was the only auxin that increased sporophore numbers over a wide range of concentrations.

Similarly, GA_3 and K reduced the total yield, primordium and sporophore numbers at high concentrations. But both hormones slightly stimulated primordium initiation when added at low concentrations.

Tested auxins were more effective in increasing the

stipe length and pileus diameter than gibberellin and cytokinin.

At some concentrations of tested phytohormones the protein content of sporophores increased significantly, but there was no consistent relationship between the protein content and phytohormone concentration applied.

In general, the shape of sporophore was not affected for all phytohormone treatments, except the size and the average weight of the sporophore were affected.

The combination of IAA, GA_3 and K slightly reduced the total yield and dry weight for all tested concentrations. While the protein content of sporophore increased significantly.

3.6 Primordium formation on semi-solid agar

The necessary events preceding the formation of mature fruit bodies involve the formation of initials which are visible aggregations of hyphae and the formation of structures termed primordia from some or all formed initials. These represent a swelling of the initial to form a mass of tissue in which a degree of fruit body differentiation is observable in the form

of a miniature stipe and pileus. Petri plate techniques have been used by some workers to study this transition from vegetative to reproductive growth.

The role of phytohormones in the formation of primordia was studied by adopting methods according to those outlined by Hume and Hayes (1972). In this method, a malt extract medium supports the formation of primordia and alternative procedures are adopted according to a method termed ‚Single-phase method in which one medium is used and ‚a Two-phase method in which malt extract agar is mechanically separated from a water agar using a partitioned Petri plate.

3.6.1 Single-phase method

The medium was prepared by disolving 2% malt extract (Boots Pure Drug Co. Limited, Nottingham) in deionized water and then 1.5% Oxoid agar No. 3 was added. The pH of the medium was adjusted to 7.5 using 0.1 N sodium hydroxide before autoclaving. After autoclaving, the medium was dispensed in 25 ml quantities into 9-cm Petri plates of the standard type. All the plates were inoculated centrally following the procedure presented in section 2.2.1 and incubated for fifteen days at 25^{o}C.

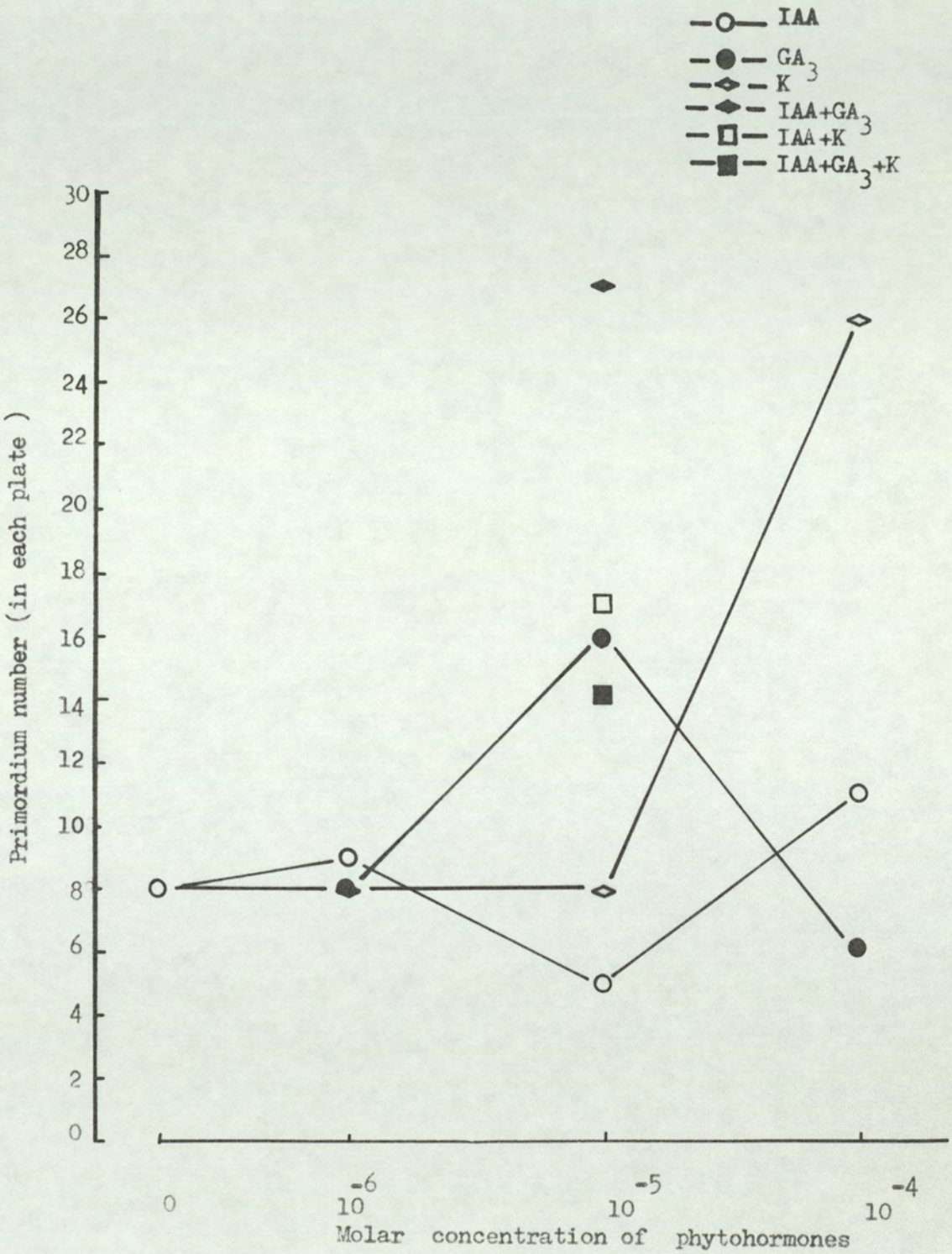
Four water agar discs containing the phytohormones were placed at the edge of each colony. Water agar discs without hormones served as control. Water agar discs were prepared by adding 1.5% Oxoid agar No. 3 in deionized water. After autoclaving water agar, sterilized hormones were added at 45°C before solidification. After setting the discs were removed using a No. 3 corkborer.

After adding water agar discs, all plates were re-incubated at the same temperature for a further five days, when the temperature was dropped to 17°C. Observing and counting primordia were carried out at three-day intervals.

For all treatments in which phytohormones were added in different concentrations, primordia appeared five-seven days earlier than the control. Also, primordium numbers increased for many treatments, but the most significant was obtained at a concentration of 10^{-4}M of K and with a combination of IAA with GA_3 at a concentration of 10^{-5}M (Table 2.5, Figure 3.33, Appendix 2.7).

There was no consistent relationship between primordium number and phytohormone concentration applied. However, it seems that there is an optimum concentration

Figure 3.33 :Effect of phytohormones on primordium formation on malt extract agar (single-phase).



of each hormone for primordium formation (Figure 3.33).

In the control, primordia were normally distributed over the surface of the agar, while in the treatments they concentrated near to the applied water agar discs (Plate 2.1). This generally restricted their growth compared to the control treatments.

Table 2.5 The optimum concentration of phytohormones for primordium formation on malt extract (single-phase method)

Concentration	Primordium number	standard error
0	8	2.7
10^{-4}M K	* 26	5.5
10^{-5}M IAA & 10^{-5}M GA_3	* 27	6.5

3.6.2 Two-phase method

Two media were used in this experiment, namely,

a - 2% malt extract agar (vegetative substrate)

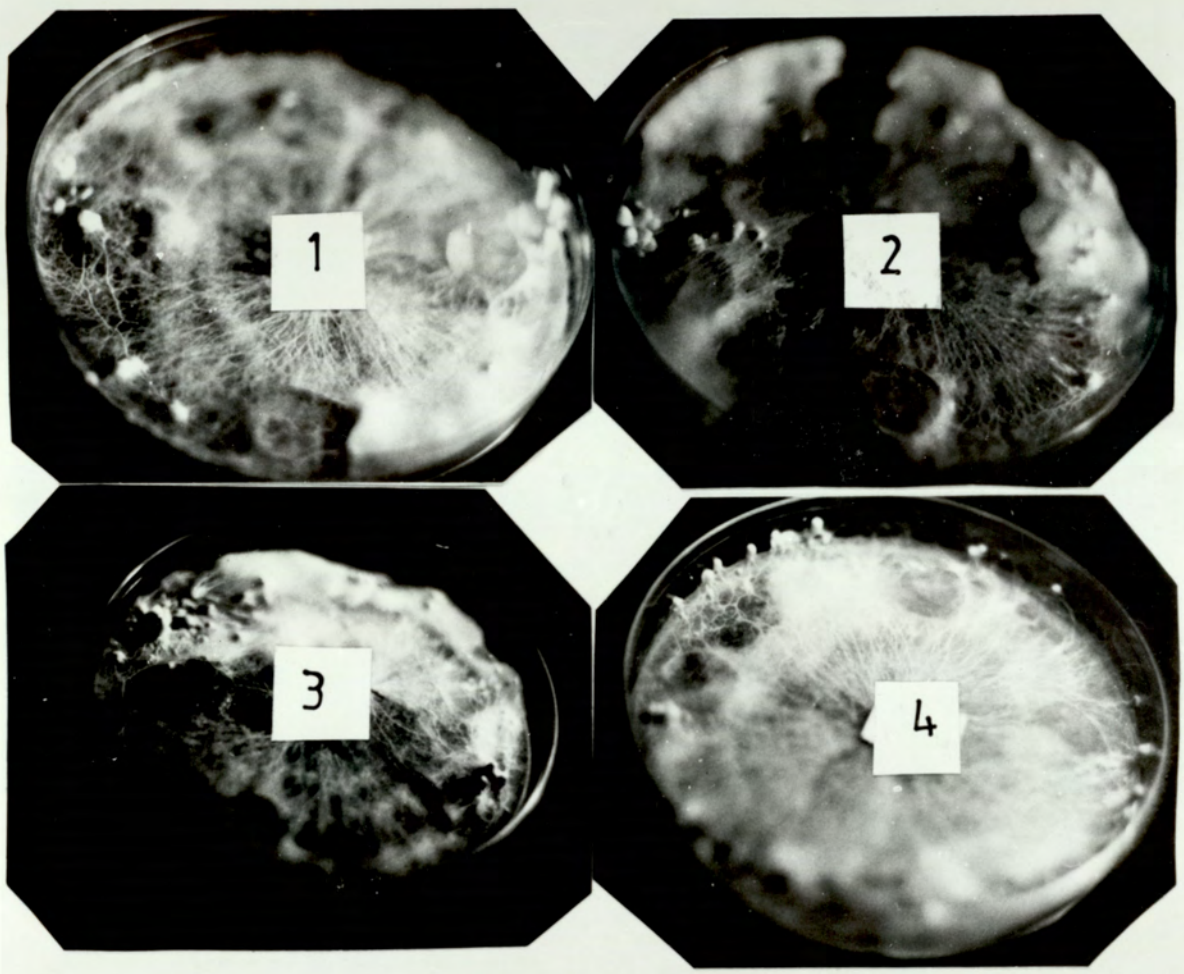
b - water agar (reproductive substrate)

15ml of 2% malt extract agar medium was poured into one half of a partitioned Petri plate. After solidification the plates were inoculated with A.bisporus mycelium and incubated for eleven days at 25°C.

15ml of water agar containing the test phytohormones at certain concentrations were poured into the second

* Significant at p=0.05 .

Plate 2.1 :Effect of phytohormones on primordium formation on malt extract agar (single-phase) .



1: Control (malt extract agar inoculated with A.bisporus mycelium) .

2: Malt extract agar supplemented with 10^{-5} M K ,10^{-5} M IAA and 10^{-5} M GA_3 .

3: Malt extract agar supplemented with 10^{-5} M IAA and 10^{-5} M GA_3 .

4:Malt extract agar supplemented with 10^{-6} M IAA .

division of the Petri plate. 15ml of water agar containing no phytohormones were poured into the second division of the control Petri plates. All plates were re-incubated for seven days at the same temperature. Subsequently, the temperature was dropped to 17°C when the mycelium colonized nearly half the water agar phase. Five replicates were prepared for each treatment. Observing and counting primordia were done at three-day intervals.

For all treatments, the total primordium numbers increased over the control, but the most significant increase was obtained with the combination of IAA with GA_3 at concentration of 10^{-5}M (Table 2.6, Figure 3.34, Appendix 2.8). Primordia formed on both media and there was a distinct difference in the distribution of formed primordia according to treatment. In control treatments, containing no added phytohormones, primordia generally formed near to the central partition, while in treatments containing added phytohormones, primordia formation also occurred at the extremities of the developing colony (Plate 2.2). This indicates that both media in the treatment series of plates contained substances which contributed to primordia formation.

The results also indicate that the optimum hormone

Figure 3.34 :Effect of phytohormones on primordium formation on malt agar (two-phase method) .

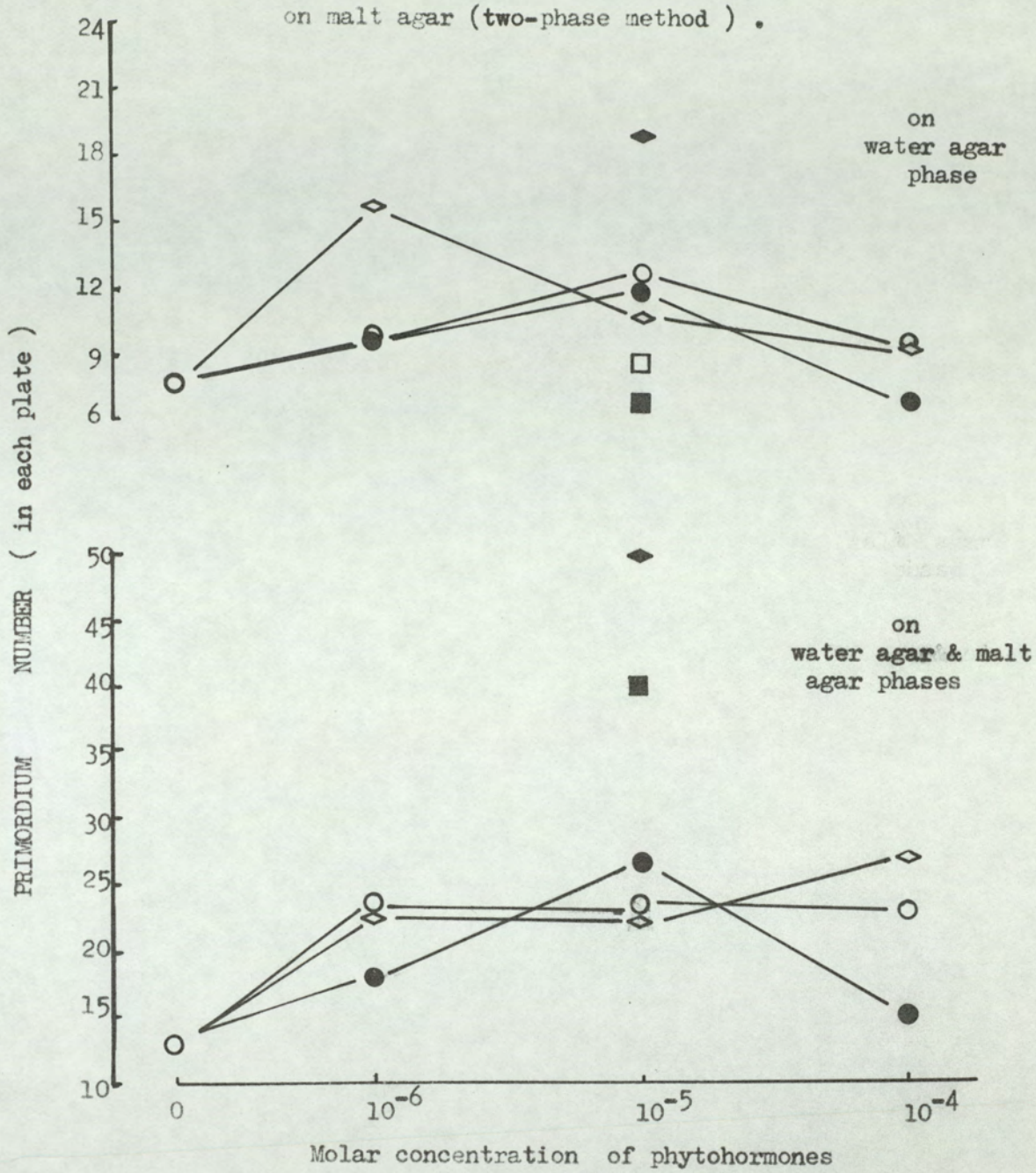
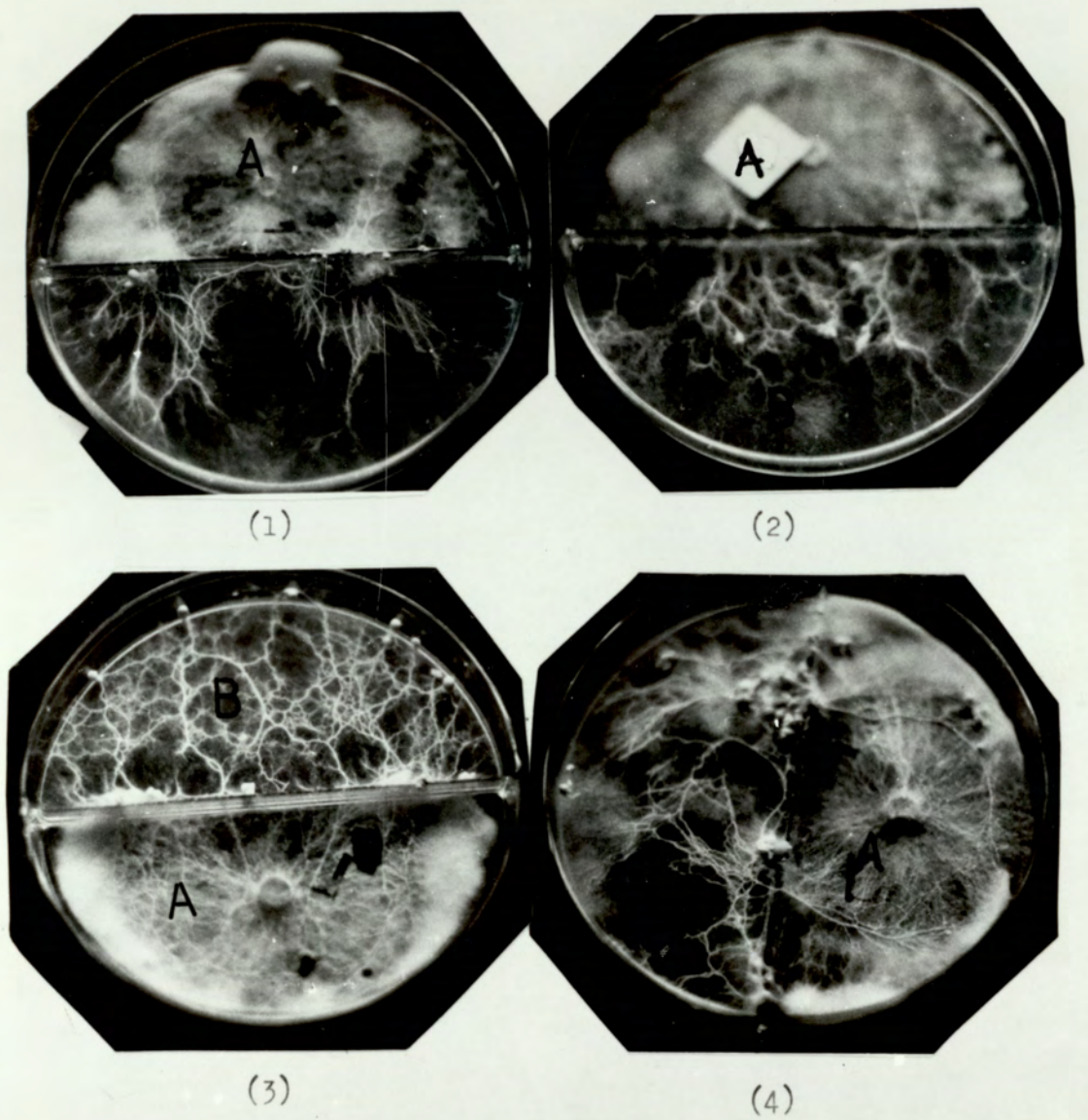


Plate 2.2 :Effect of phytohormones on primordium formation on malt extract agar and water agar (two-phase) .



1: Control (A: malt extract phase ,B:water agar phase).

2:Water agar phase (B) supplemented with 10^{-4} M IAA .

3:Water agar phase (B) supplemented with 10^{-6} M K .

4:Water agar phase (B) supplemented with 10^{-5} M IAA & 10^{-5} M GA_3.

concentration for primordium formation was 10^{-5}M, except for kinetin which produced maximum number at 10^{-6}M.

Table 2.6 The optimum concentration of phytohormones for primordium formation on water agar phase (two-phase method)

Treatment	Primordium number of water agar phase	Standard error
0	8	1.8
10^{-5}M IAA	* 13	2.0
10^{-5}M IAA & 10^{-5}M GA_3	* 19	5.3

3.6.3 Conclusions

From these results it may be concluded that phytohormone addition to malt extract influences the number of primordia formed using semi-solid plate techniques. Although no consistent relationship between primordia number and phytohormone concentration could be found within the range used in this study an optimum concentration was definable. Combinations of IAA and GA_3 at 10^{-5}M were particularly effective.

It may be concluded from these investigations that malt extract contains substances which are stimulatory to primordia formation and that by the add-

* Significant at p=0.1.

ition of some phytohormones numbers are increased, suggesting an addition effect. Similar addition effects were shown when phytohormones were added to water agar, located alongside the vegetative malt extract medium.

In order to test the possibility that the stimulatory substances in malt extract were phytohormones , tests were done according to procedures outline in sections 2.7, 2.8, and 2.9, these tests confirmed that malt extract contains IAA, gibberellin, and cytokinin at high concentrations, namely, 9.133, 11.693, and 20.50 ppm, respectively (Appendix 2.9).

Part III ENDOGENOUS PRODUCTION OF PHYTOHORMONES BY A.BISPORUS

A necessary first step in studying the role of physiologically active substances in the growth and development of an organism is determine its production and utilization during the various growth stages.

This part of the study was designed to determine the capacity of mycelium to produce and utilize phytohormones in liquid culture and to estimate quantitatively phytohormones in mycelium and the tissues of the fruit body.

3.7 Phytohormones and vegetative growth

3.7.1 Phytohormones and mycelium:

Two samples (15 - 20g fresh weight) from a four-week old culture were used for each hormone analysis according to methods outlined in sections 2.7., 2.8., and 2.9. Also, three samples (1 - 2g fresh weight) of the mycelium were used to determine the dry weight.

Three groups of phytohormone were identified in the A.bisporus mycelium, namely, auxins, gibberellins and cytokinins. The extracted auxin was found to be mostly IAA.

A.bisporus mycelium contained very low concentration of IAA compared to gibberellin and cytokinin (Table 3.1).

Table 3.1

Phytohormone concentration (ppm/dry weight) in mycelium of A.bisporus

Samples	IAA	Gibberellin	Cytokinin
1	0.0025	1.69	9.035
2	0.001	6.55	3.05
Mean	0.00175	4.12	6.042
Standard error	0.00075	2.43	2.99

3.7.2 Phytohormone production by A.bisporus mycelium

A.bisporus was cultivated in liquid culture (section 2.3), using the synthetic medium (section 2.2.2) in 150ml flasks (each one containing 50ml).

Some flasks were left without vitamin additions, others were only supplemented with biotin, thiamin or ethyl acetate. The control flasks were left without inoculation.

Three flasks were harvested at weekly intervals and the filtrates were prepared for hormone analysis

(section 2.7., 2.8., 2.9).

The concentration of IAA in the culture medium reached a maximum after two weeks of incubation and then declined after three and four weeks (Fig. 3.35).

From R_F values on thin layer chromatograms (Plate 3.1) and paper chromatograms, the Ehrlich reaction, the Salkowski reaction, the absorption spectrum for the Ehrlich colour complex (Plate 3.2), pea root bioassay, and cress root bioassay, indicate that the auxin produced by A.bisporus mycelium was mostly IAA. An unknown pink spot appeared with Ehrlich's reagent between the starting point and the IAA spot on chromatograms (see Plate 3.1).

Also, chromatographic analysis show that synthesis of IAA is not vitamin dependent (see Plate 3.1).

Similarly, the concentration of gibberellin increased as the period of incubation was increased and reached a maximum after two weeks of incubation, then declined sharply after three and four weeks of incubation (see Fig 3.35).

The results of lettuce bioassay show that most gibberellin activity appears at R_F 0.2-0.6 that corresponding to the range of synthetic GA_3 activity.

Figure 3.35: Phytohormones production by A.bisporus mycelium .

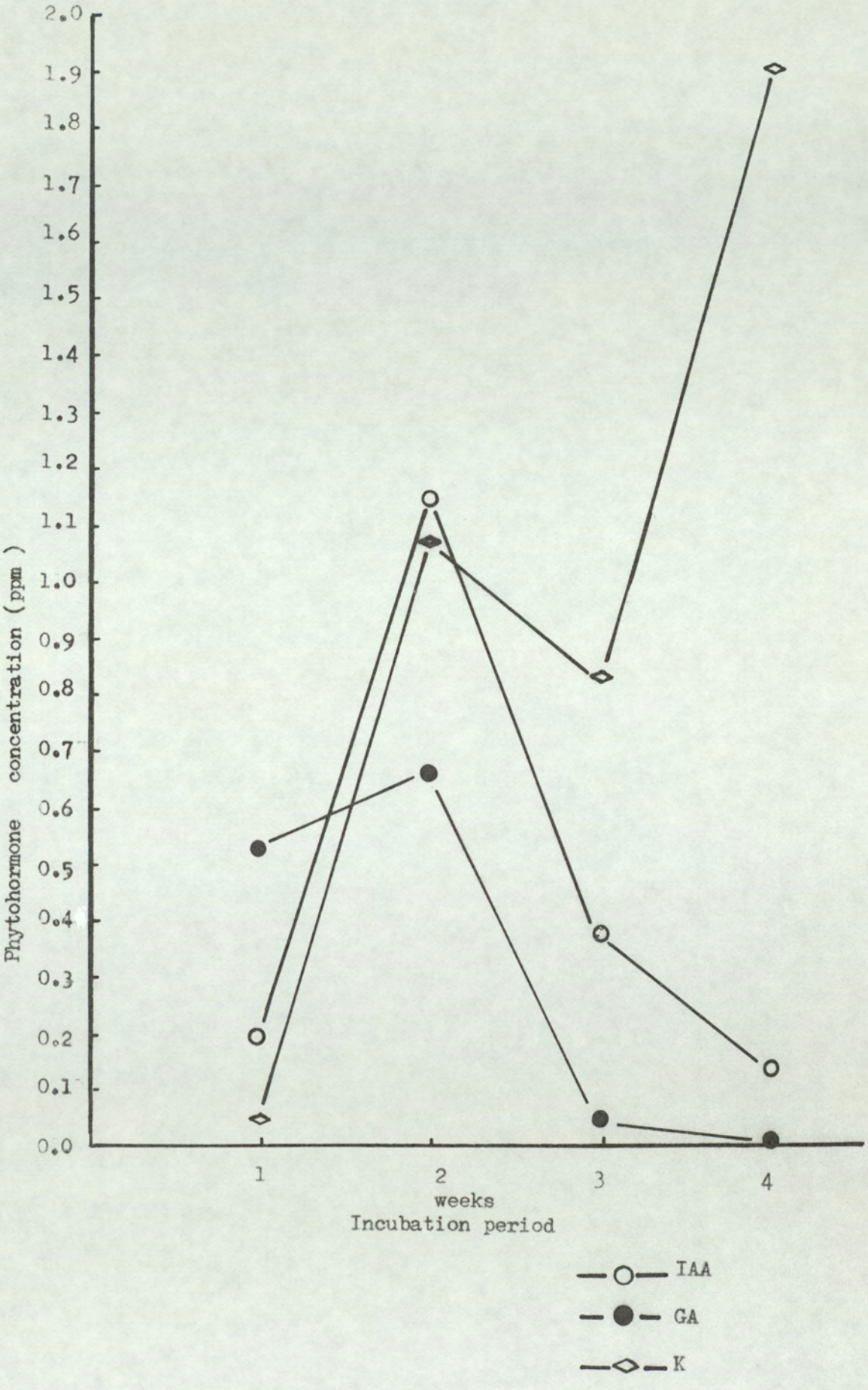


Plate 3.1 :One -dimensional thin-layer chromatography of variuos culture filtrates of Agaricus bisporus

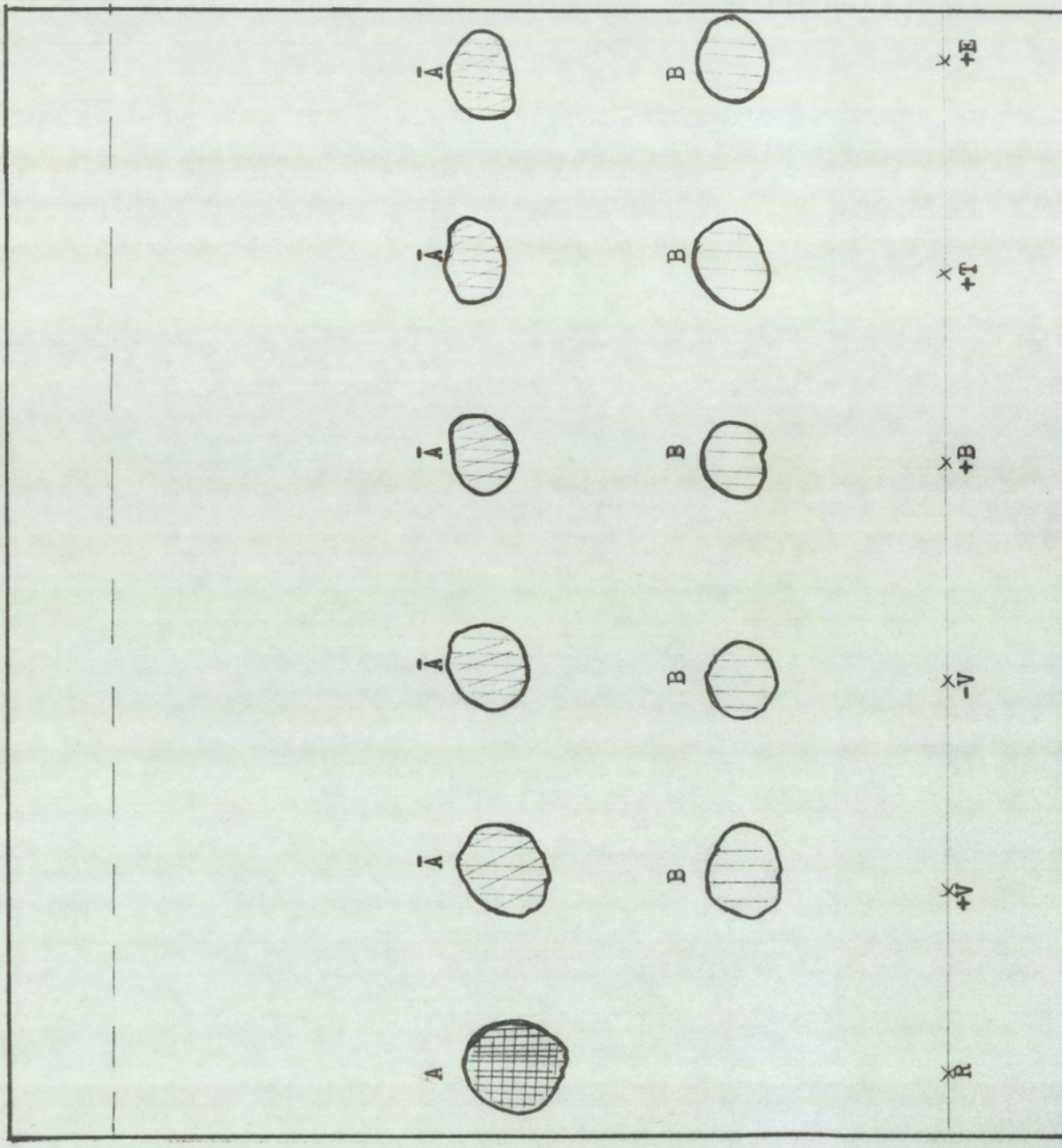


Plate 3.1 (continued.....)

The chromatogram was developed with methyl acetate ,isopropanol, ammonia (45:35:20) and treated with Ehrlich's reagent .

R=synthetic IAA .

+V=extract from A.bisporus culture medium supplemented with biotin, thiamin and ethyl acetate.

-V=extract from A.bisporus culture medium without vitamin additions.

+B=extract from A.bisporus culture medium supplemented with biotin only.

+T=extract from A.bisporus culture medium supplemented with thiamin only.

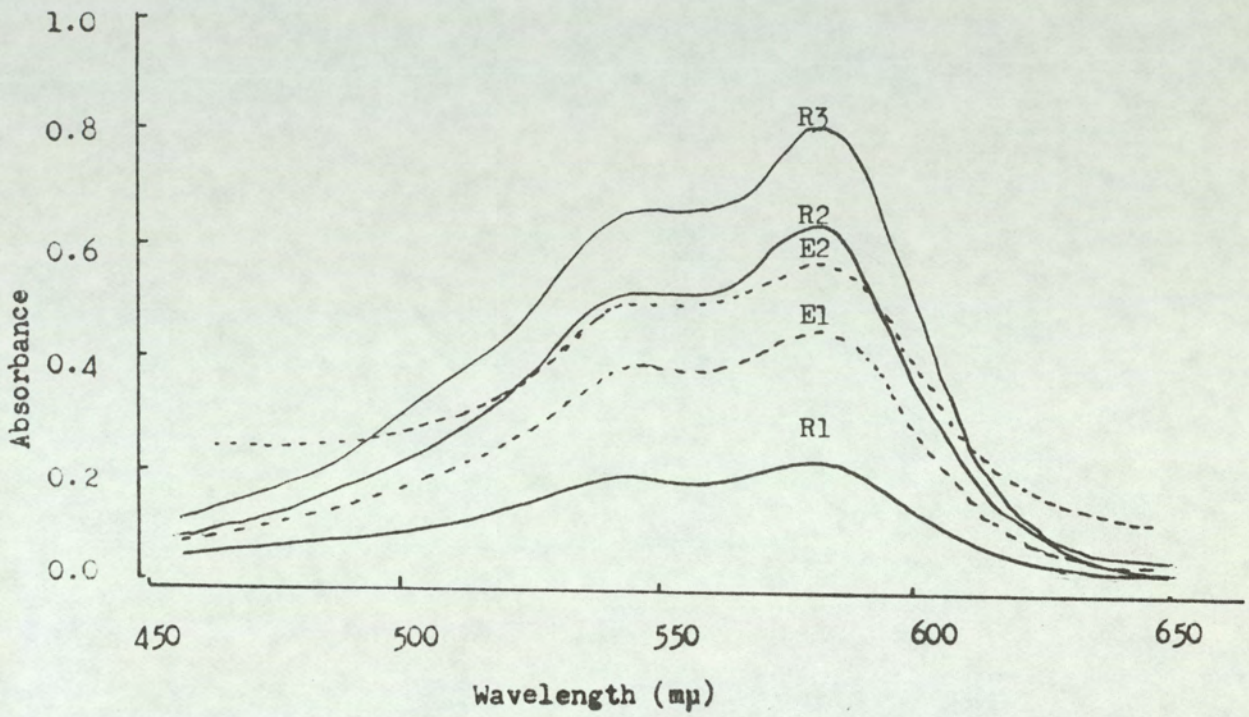
+E=extract from A.bisporus culture medium supplemented with ethyl acetate only.

A=blue-purple reaction with synthetic IAA .

$\bar{A}$ =blue-purple reaction developed with the extracts .

B=pink reaction appeared between starting point of extract and IAA spot .

Plate 3.2 : Absorption spectra for Ehrlich colour complex formed with synthetic IAA and extract from culture filtrate of A.bisporus .



R1= 10 μg of standard solution of synthetic IAA .

R2=30 μg of standard solution of synthetic IAA.

R3=70 μg of standard solution of synthetic IAA .

E1=120 μl of concentrated extract obtained from 400 ml of culture filtrate .

E2=650 μl of concentrated extract from 400 ml of culture filtrate.

Also radish cotyledon bioassay showed that A.bisporus has the ability to synthesize and secreted cytokinin in the culture medium. As with auxin and gibberellin, cytokinin production was very low after the first week of incubation, and reached a maximum after the second week, declined after the third week, but increased again sharply, after the fourth week of incubation (Fig. 3.35, Appendix 3.1).

The cytokinin activity was mostly detected at R_F 0.6 which corresponded to the range of kinetin activity.

3.7.3 Phytohormone utilization by A.bisporus

In order to test the ability of A.bisporus to utilize synthetic hormones, IAA, GA_3 and K, a 10 ppm concentration of these hormones solutions were added to liquid culture. All the flasks were inoculated with A.bisporus mycelium, except those of the control, which were left without inoculation. Three flasks from each treatment were harvested and the filtrates were prepared for hormone analysis (section 2.7., 2.8., 2.9). Also the dry weight of the mycelium was determined.

The concentration of added IAA decreased as the period of incubation was increased, while the greatest losses occurred after the first and second week of

incubation (Fig 3.36, Appendix 3.2). This was also associated with high increase in the dry weight of harvested mycelium compared with that of the control (without auxin addition) (Fig 3.37).

Similarly, A.bisporus mycelium utilized GA_3 and K, and the greatest losses occurred after the first and second week of incubation (Fig. 3.36). The losses of both GA_3 and K were also associated with an increase in the dry weight of the harvested mycelium (Appendix 3.2).

3.8 Phytohormones and reproductive growth

3.8.1 Phytohormones and different stages of sporophore development.

A.bisporus sporophores grown on compost were harvested at different stages of development and extracted for hormone analysis (section 2.7., 2.8., 2.9.) Two samples of each stage (30 - 40g fresh weight) were used for each hormone analysis. The following stages were selected:

a - Primordium stage, 2-day old.

b - Button stage, 3-day old.

c - Cup stage, 5-day old.

d - Flat stage, 7-day old.

All tested stages contained a considerable amount

Figure 3.36 : Phytohormone utilization by A.bisporus mycelium .

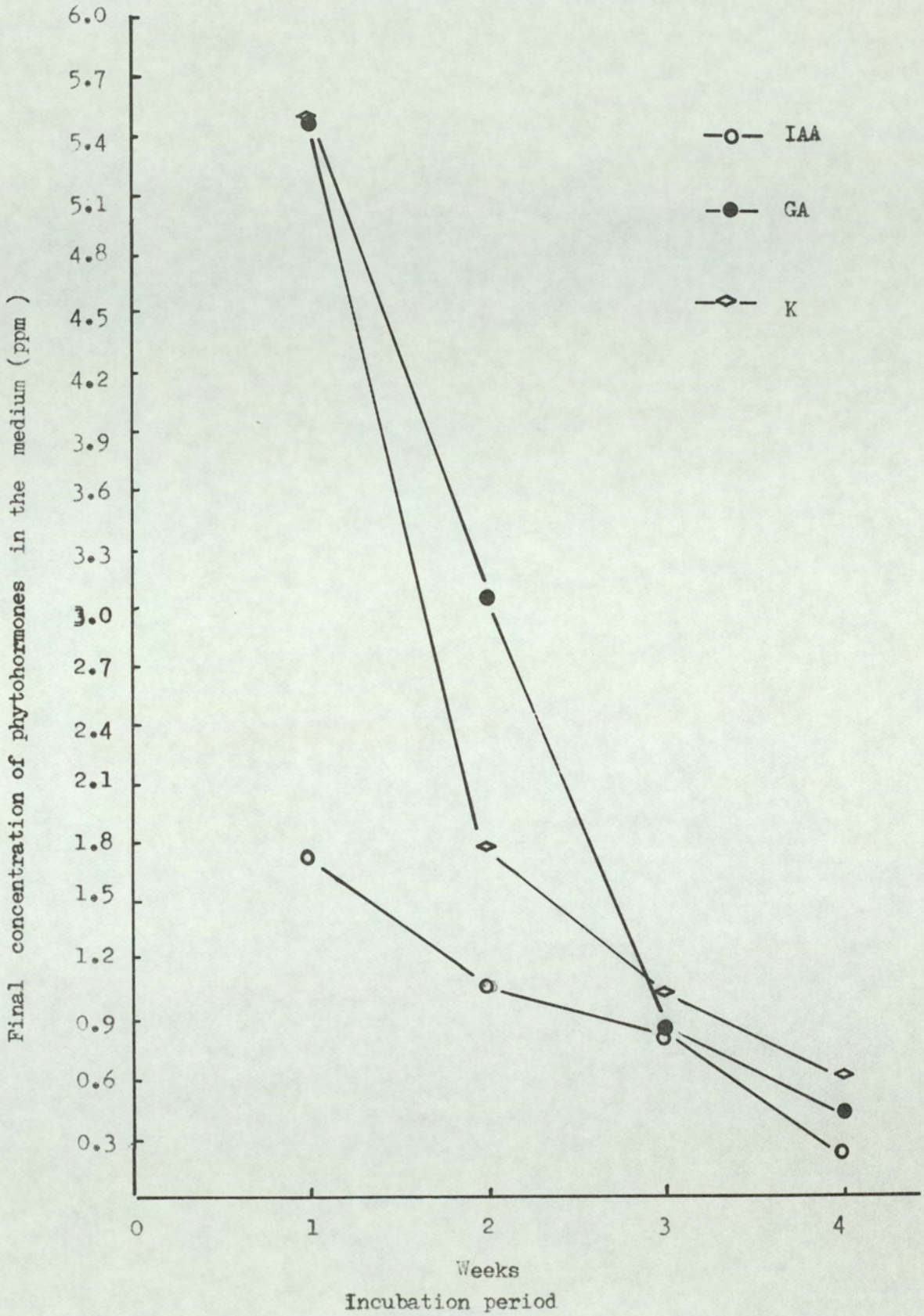
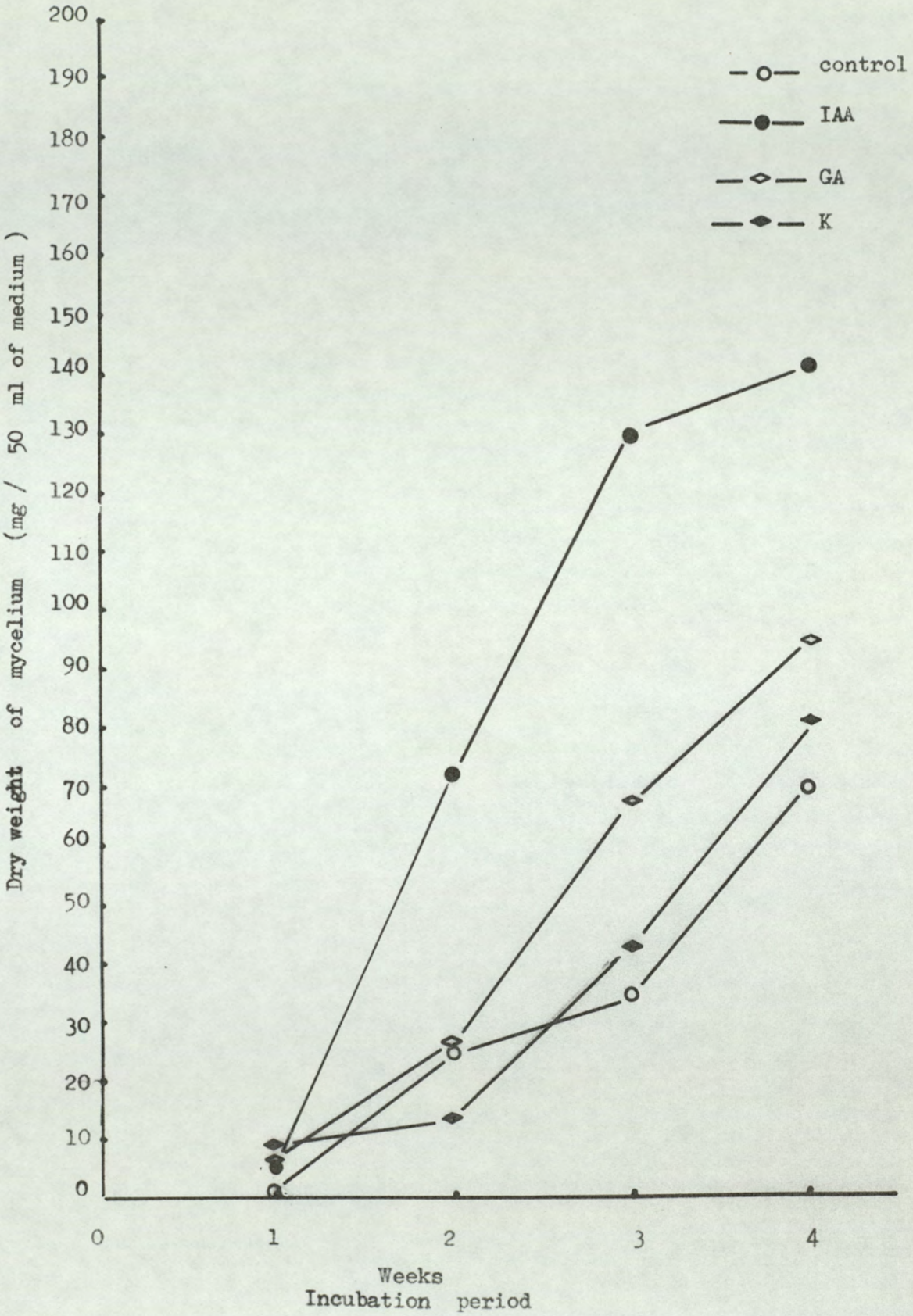


Figure 3.37:Effect of phytohormones on mycelial growth in liquid culture .



of auxin, gibberellin and cytokinin. The auxin extracted from the sporophores seemed to be mostly IAA. Also, the pink spot, as with mycelium and culture medium analysis, appeared between the starting point and IAA spot on the chromatograms.

The results (Appendix 3.3) show that an inverse correlation between IAA content and the age of the sporophore (Fig 3.38). Thus the highest level of IAA was detected in the primordium stage, while the lowest level was at the flat stage.

Similarly, there was an inverse correlation between the amount of gibberellin and the age of the sporophore (Fig. 3.39, Appendix 3.3). Thus the primordium stage also contains the highest concentration of gibberellin detected, while the flat stage containing the lowest concentration.

Also, gibberellin activity appeared at R_F 0.2 - 0.6. This indicates the similarity between the gibberellin detected in the mycelium, in the culture medium and sporophores.

As with auxin and gibberellin, there is an inverse correlation between cytokinin content and the age of the sporophore, but the amount of cytokinin reached a maximum at the button stage and then declined as the

Figure 3.38 : Correlation between IAA content and fruit body age .

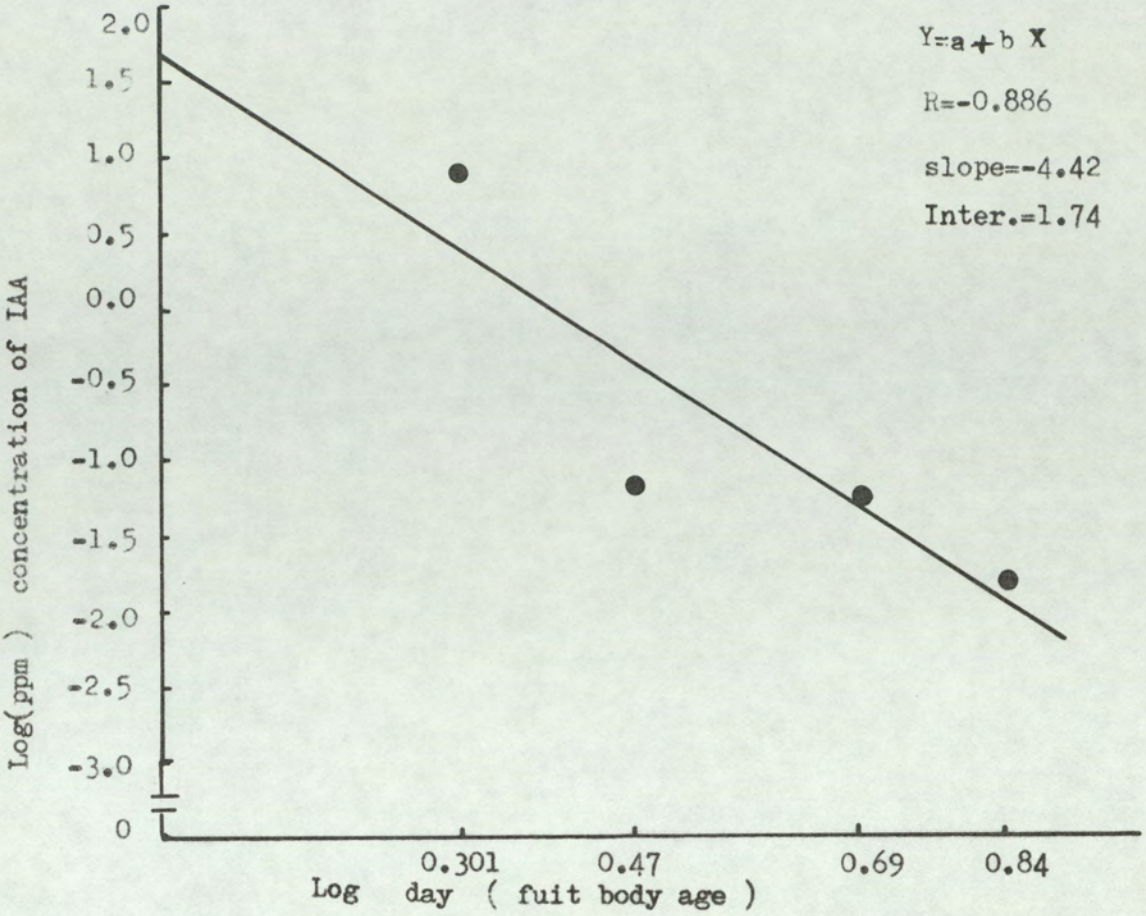
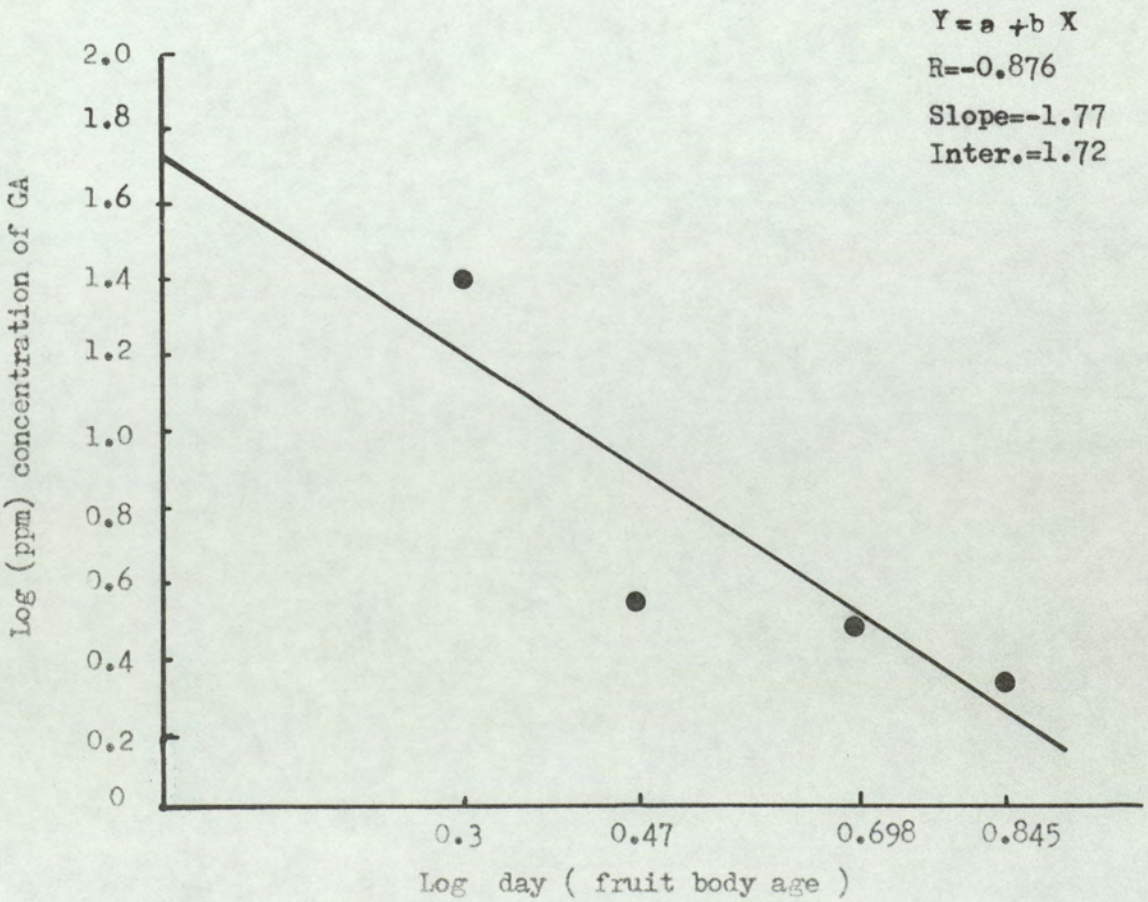


Figure 3.39 :Correlation between GA content and fruit body age.



sporophore age was increased (Fig 3.40).

Cytokinin activity was detected also at R_F 0.6 which is similar to those detected in the mycelium and culture medium. This indicates the similarity between the cytokinin in the mycelium, sporophore and culture medium.

3.8.2 Phytohormones and different parts of the sporophore

Sporophores at the cup stage (stipe length 35 - 45mm and cap diameter 35 - 40mm) were selected as a standard for extraction of phytohormones and the following parts of the sporophore were selected:

a - Gills.

b - Pilei (gills removed).

c - Stipes.

Extraction, identification and estimation of phytohormones were carried out according to the standard methods in sections 2.7., 2.8., 2.9.

Two samples from each part (30 - 40g fresh weight) were used for each hormone analysis.

The highest concentration of IAA was detected in the stipe, while the gills contained the lowest level. Thus there was a significant difference between IAA content in gills and stipes (see Fig 3.41, Appendix 3.4).

Figure 3.40 : Correlation between K content and fruit body age .

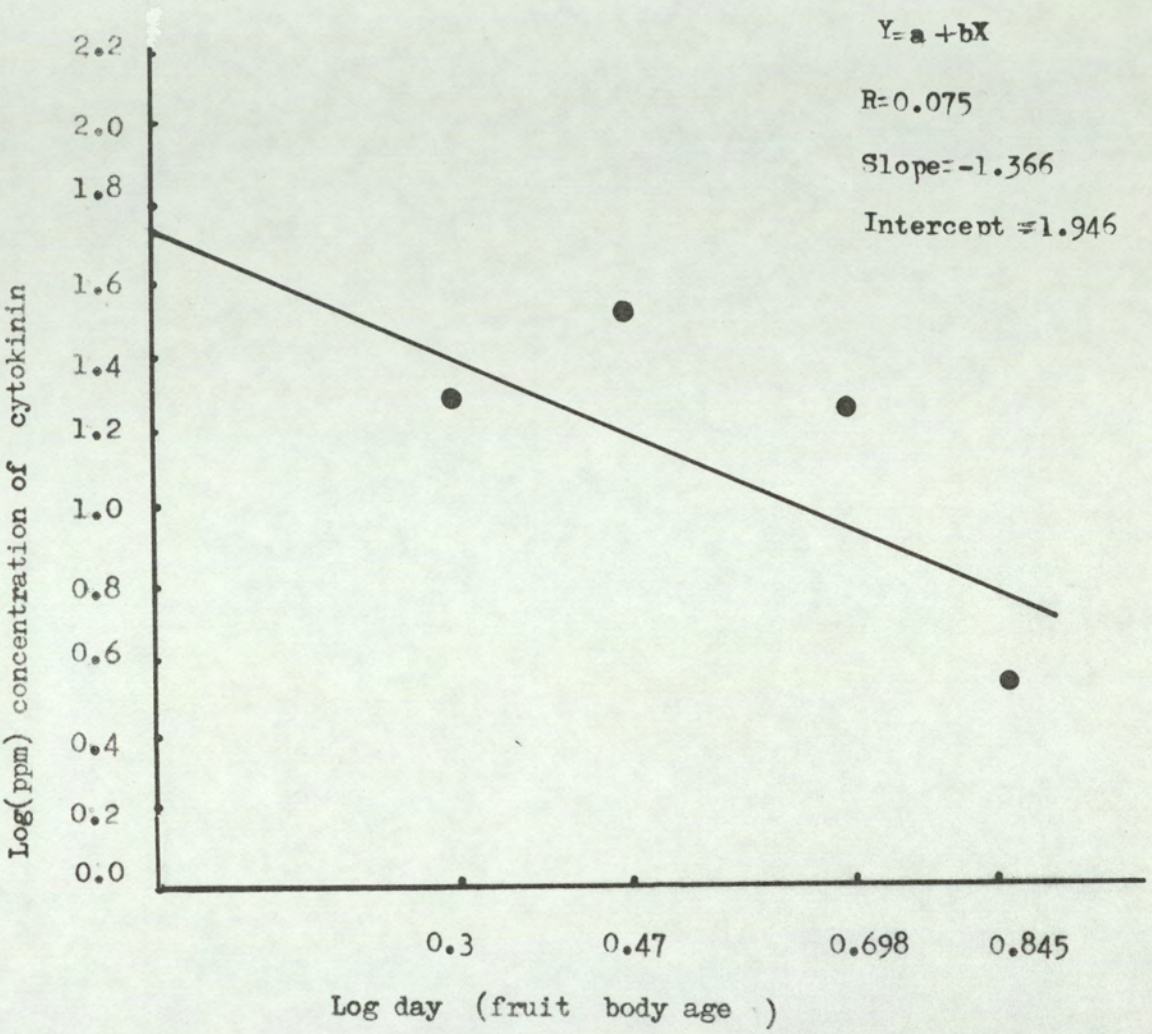
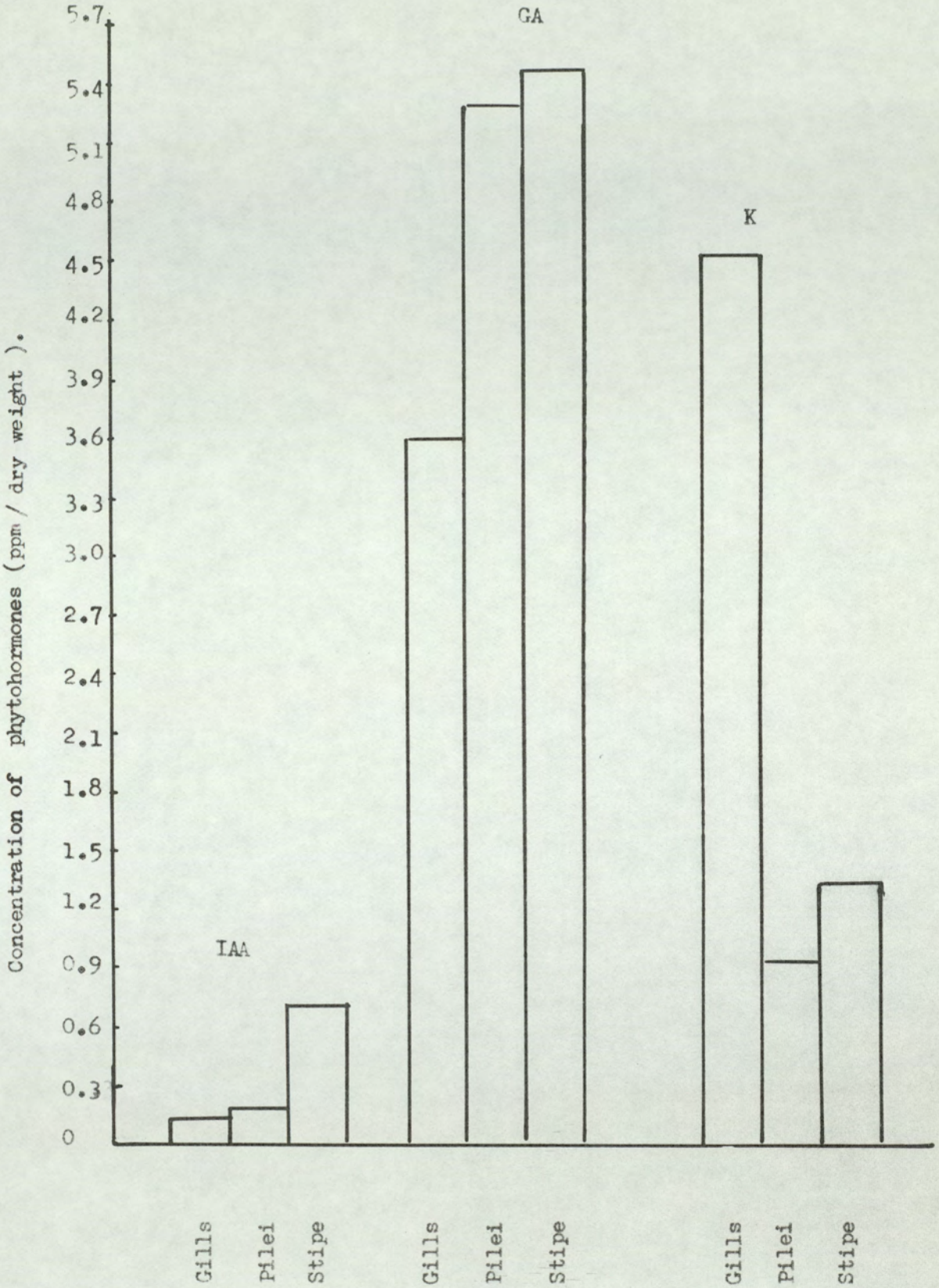


Figure 3. 41 :Distribution of phytohormones in different parts of the sporophore .



3.9 Conclusions

Considerable amounts of three phytohormones (auxin, gibberellin, and cytokinin) were detected in the mycelium and fruit bodies of A.bisporus. In general, their level in the mycelium was less than that in the fruit bodies. While the highest level was detected in the primordium stage. Moreover, there was an inverse correlation between phytohormone content and different stages in the development of sporophore. This indicates clearly the sharp change in the phytohormone level during the transition from mycelial growth to reproductive growth.

A.bisporus mycelium was able to produce the three phytohormones from the synthetic ingredients, and their level reached a maximum after two weeks of incubation and declined as the period of incubation was increased.

Also, the mycelium utilized the synthetic phytohormones significantly and the losses of added phytohormones were associated with increases in the dry weight of the harvested mycelia. This indicates that A.bisporus utilized phytohormones as carbon sources or as promoting growth substances. Thus, phytohormones are not major-limiting factors, since the mycelium has the ability to synthesize them, even from a simple synthetic medium.

Part IV PHYTOHORMONES IN NATURAL SUBSTRATES

In the methods adopted in commerce two substrates, a compost and a casing soil are used, the former supporting the vegetative stage of growth and the latter the reproductive stage. Both substrates are selective in that they permit A.bisporus to colonise at the expense of other competitor fungi. However, other micro-organisms are present and active during both vegetative and reproductive stages of growth. Thus, the culture system may be regarded as a mixed culture.

Studies on the microbial ecology of compost preparation and subsequent colonization by Hayes (1968) and Stanek (1972) suggest a strong relationship between the micro-organisms and growth of A.bisporus and their work indicates a close interrelationship between bacteria and growth. Similarly, Hayes et al (1969), Hayes (1972) and Cresswell and Hayes (1979) have studied the interrelationship between bacteria and the formation of fruit bodies in the casing layer. Certain bacteria, in particular isolates of gram negative Pseudomonads, have been shown to be essential for fruit body formation to take place. Giovannozi-Sermanni et al (1974) implicated the microbial composition of compost with the fluctuation in level of phytohormones.

In view of the physiological effects of phytohormones on different aspects of A.bisporus growth found in this study, it was thought useful to determine the levels of phytohormones in both compost and casing soil during the course of vegetative and reproductive growth and establish any possible relationships between phytohormones and the population levels of bacteria and fungi which are associated with A.bisporus when growing in compost and casing soil.

3.9 Phytohormones in compost and casing soil

Two, 100g fresh weight samples of compost and casing soil were used for hormone analysis (section 2.7., 2.8., 2.9) at the following intervals:

a - Compost before inoculation, day 0.

b - Compost after complete colonization by A.bisporus, day 18.

c - Casing soil before application, day 18.

d - Compost and casing soil 10 days after casing, day 28.

e - Compost and casing soil after 1st flush, day 45.

f - Compost and casing soil after 2nd flush, day 53.

A pasteurized compost contained both auxin and gibberellin at concentrations higher than that in casing soil. Whereas the latter contained cytokinin at higher levels than that in compost.

Auxin concentrations in compost fluctuated sharply throughout the life-cycle of A.bisporus (Fig 3.42, Appendix 4.1). Thus it declined on day 18 when mycelium had fully colonized the compost. The levels then increased in the ten days following casing application, but then again declined at the 45 day sampling time. Levels increased again at day 53 after the second flush. In contrast, levels in the casing soil were negligible in the first 10 days after application, but at completion of the first harvest high levels were obtained. By the end of the second harvest levels had declined (Fig 3.42, Appendix 4.2).

Gibberellin concentrations in the compost increased gradually and then reached a maximum on day 28 (10 days after casing application). Subsequently, it declined slightly after first and second harvest. (Appendix 4.1). In the casing soil, a different pattern was evident. Thus, the concentration increased sharply and then reached a maximum on day 28 (10 days after casing application) and then declined strongly after the first harvest (day 45). Subsequently, it increased again after the second harvest (day 53) but to a lower level than on day 28 (Fig 3.43, Appendix 4.2).

The fluctuation in cytokinin levels in compost was similar to that of gibberellin levels. Thus, cytokinin

Figure 3.42 : Auxin concentration in compost and casing soil .

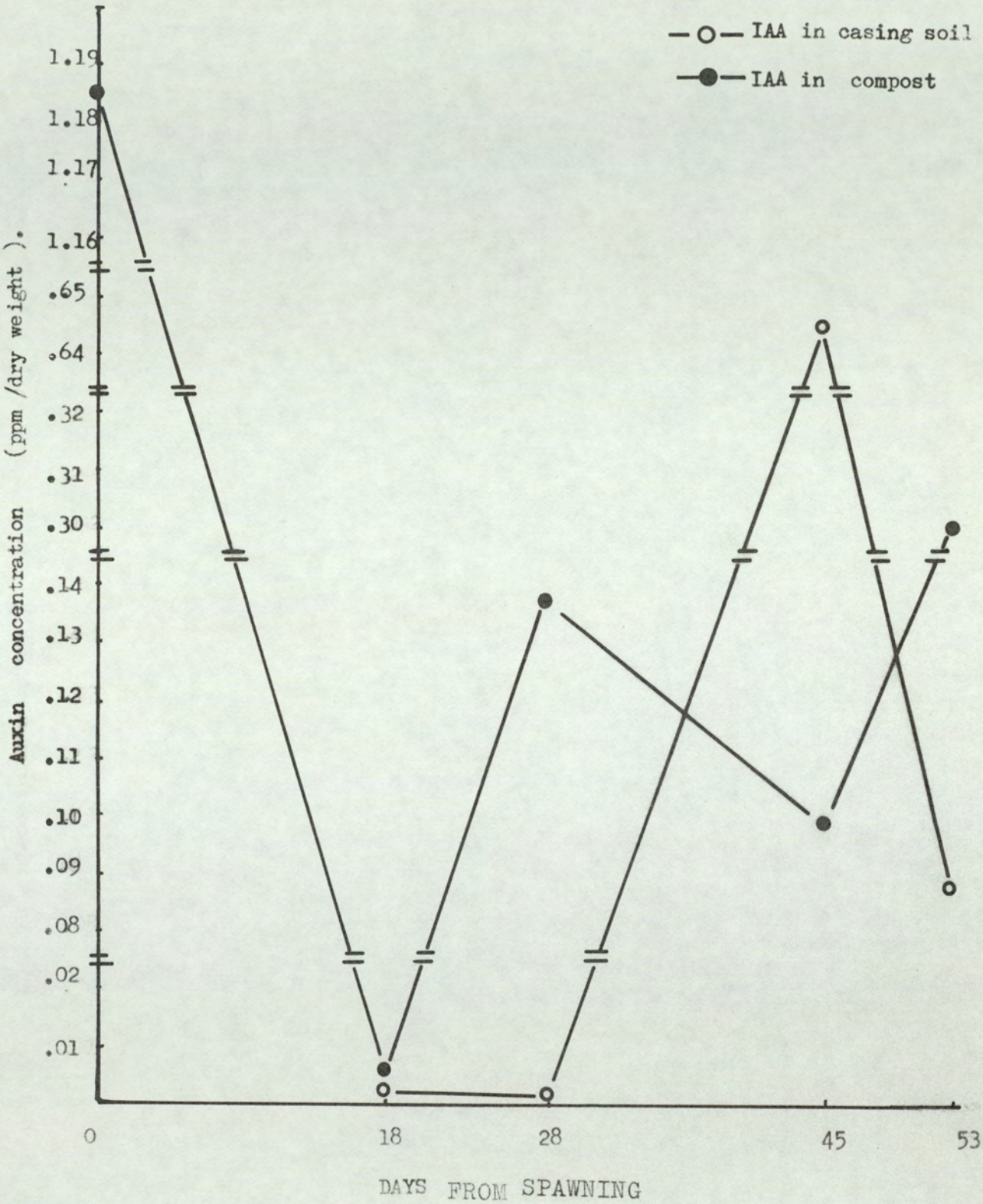


Figure 3.43 :GA concentration in compost and casing soil .

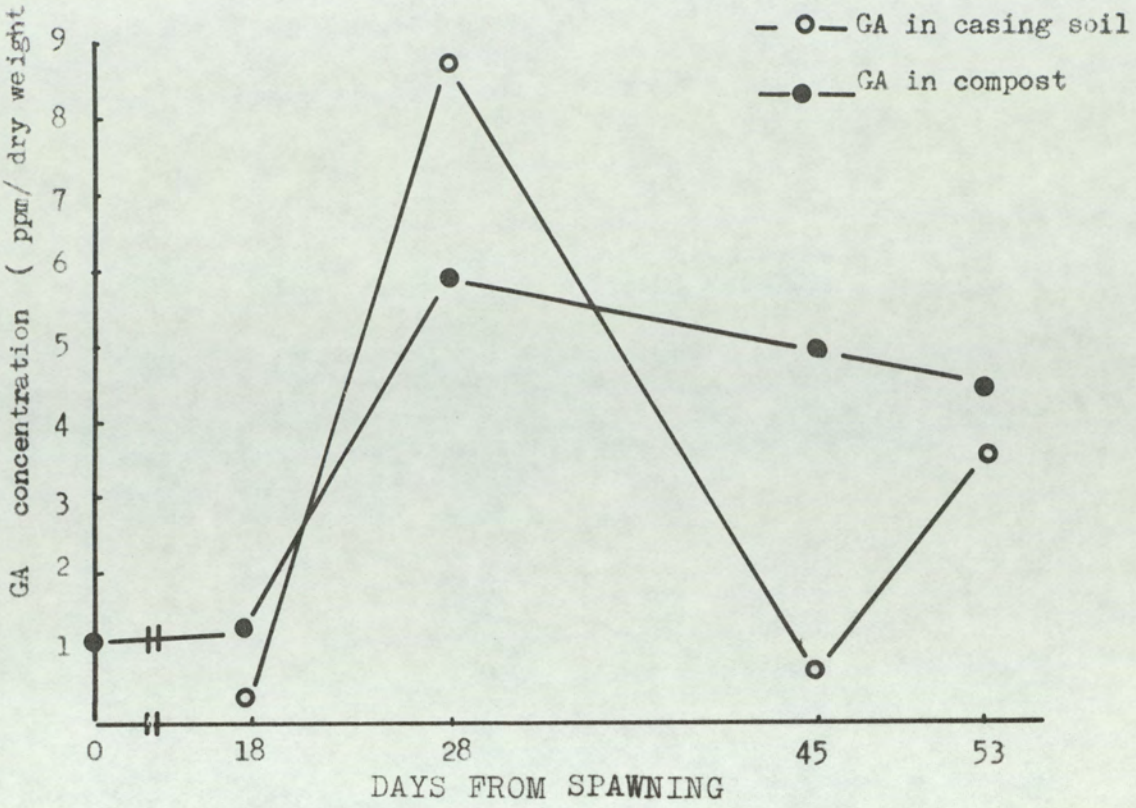
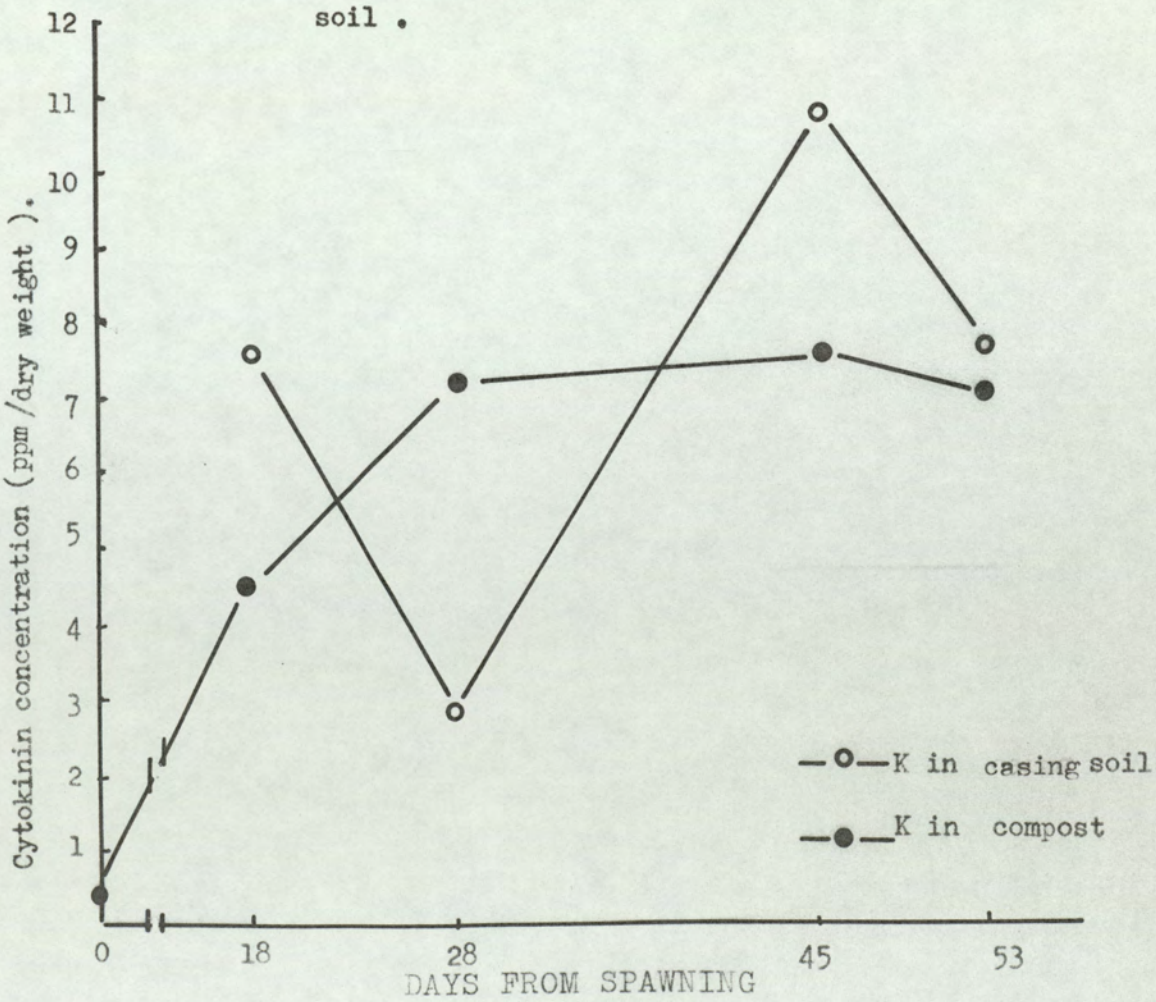


Figure 3.44 :Cytokinin concentration in compost and casing soil .



concentrations increased as the age of culture was increased (Fig 3.44, Appendix 4.1). While cytokinin in casing soil decreased significantly on day 28 and then increased again on day 45. However, the concentration decreased again on day 53 (Fig. 3.44, Appendix 4.2).

In general, the pattern of the fluctuations in each hormone concentration in the compost was opposite to that in the casing soil.

3.10 Population levels of bacteria and fungi

Estimations of the bacteria and fungal population in compost and casing soil were done according to standard procedures presented in sections 2.5.1 and 2.6.1, respectively. One gram of compost or casing soil was shaken with 100ml of 1% sterilized peptone for 10 - 20 minutes and then serial dilutions made. Total number of bacterial colonies on nutrient agar were counted after three days of incubation. While total fungal colonies on a dextrose peptone medium (section 2.6.3) were counted after seven days of incubation.

Number of bacteria, in compost, decreased significantly at day 18 after spawning, and increased to a peak 10 days after casing, after which number declined

(Appendix 4.3., Fig 3.45). From results summarized in Appendix 4.3, it was found that following casing application bacteria numbers in casing soil, increased progressively reaching a maximum after first flush but then declined after second flush (Fig. 3.45). Number of fungi in compost during the life-cycle of A.bisporus followed a similar pattern, declined 18 days after spawning and rising to a peak after casing. (Fig. 3.46, Appendix 4.4). After first and second flush the numbers declined sharply. Fluctuations in numbers of fungi in casing soil were almost opposite to that in compost (Fig 3.46). Therefore, numbers declined 10 days after casing, then rose after first flush, but declined again after the second flush.

3.11 Production and utilization of phytohormones by selected bacterial and fungal isolates.

3.11.1 Phytohormone production by isolated bacteria and fungus

A dominant bacterium in the casing soil (10 days after casing application) was selected for hormone analysis. The isolated bacterium, a gram negative, rod, was grown in submerged culture (section 2.5), and incubated at 27^{o}C on a rotary shaker. After seven days of incubation, three flasks for each hormone analysis were harvested and prepared (sections 2.7., 2.8., 2.9). The control flasks were left without inoculation.

Figure 3.45 :Population of bacteria in compost and casing soil .

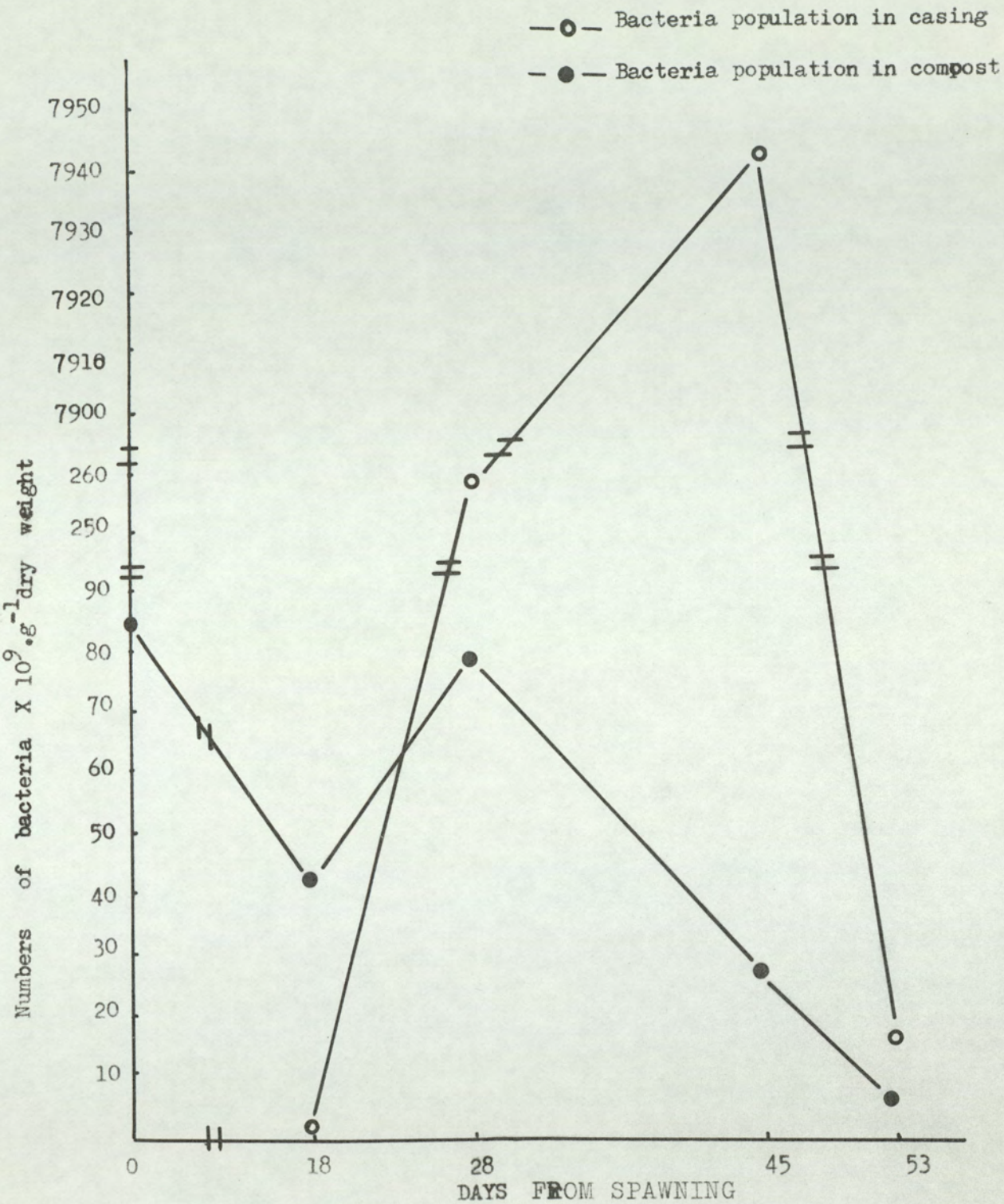
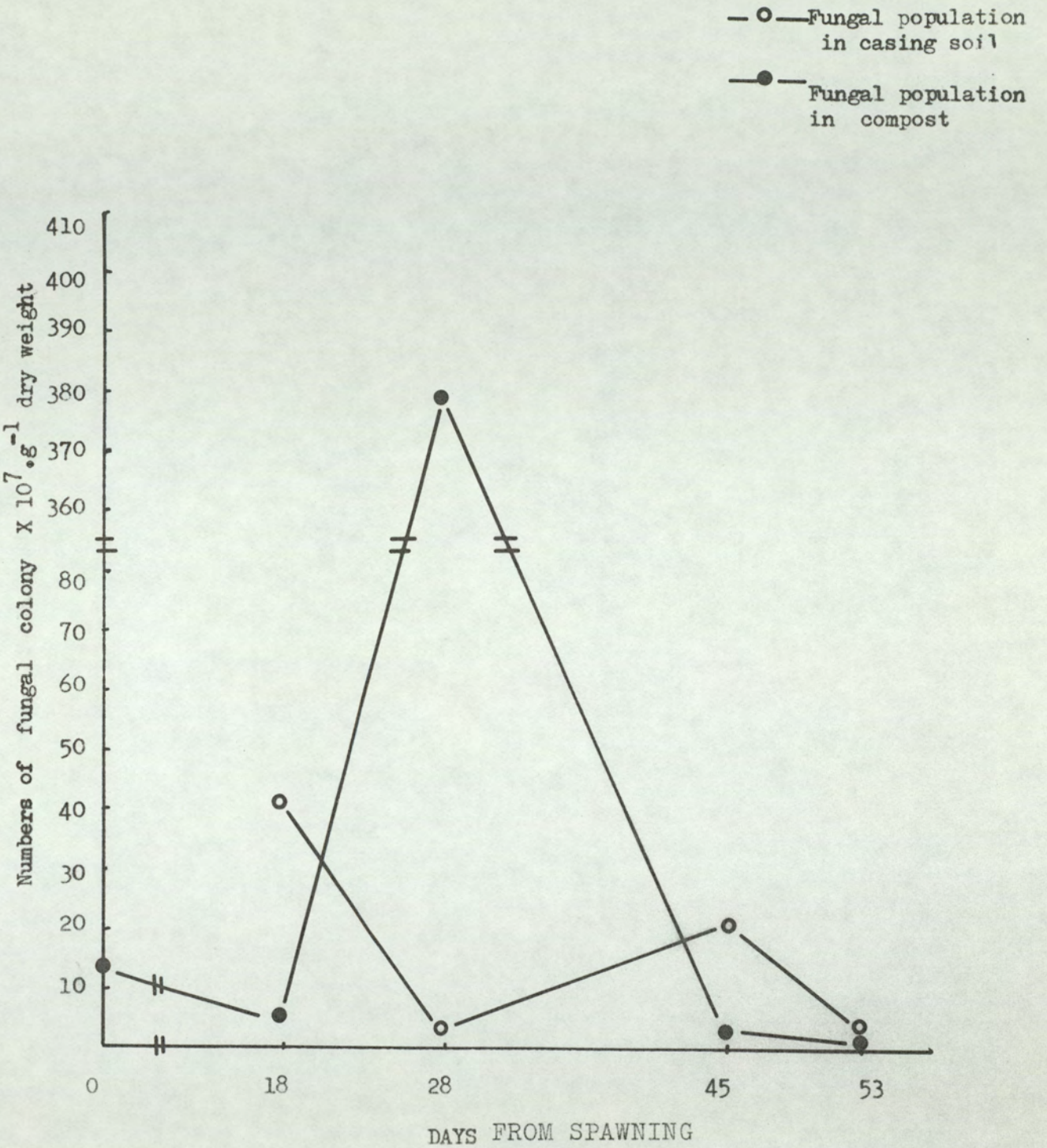


Figure 3.46 :Fungal population in compost and casing soil.



Similarly, the dominant fungus (Penicillium sps.) in the casing soil (10 days after casing application) was also selected for hormone analysis. Thus, the isolated fungus was cultivated in submerged culture using Czapek Dox medium (section 2.6). The flasks were then incubated at 25°C on a rotary shaker. After 7 days of incubation, three flasks were harvested and prepared for hormone analysis (section 2.7., 2.8., 2.9). The harvested mycelium was dried for dry weight determination. The control flasks were left without inoculation.

The isolated bacteria produced IAA at high concentrations, while the concentrations of both gibberellin and cytokinin was lower than that of auxin (Fig. 3.47, Appendix 4.5).

The isolated fungus produced auxin and gibberellin, but cytokinin could not be detected in culture fluids. (Fig. 3.48, Appendix 4.6).

3.11.2 Phytohormone utilization by isolated bacteria and fungus

The isolated bacteria was grown in submerged culture to which phytohormones were added at a concentration of 10ppm. The control flasks were left without bacteria inoculum. After seven days of incubation, three

Figure 3.47:Phytohormone production and utilization by isolated bacteria .

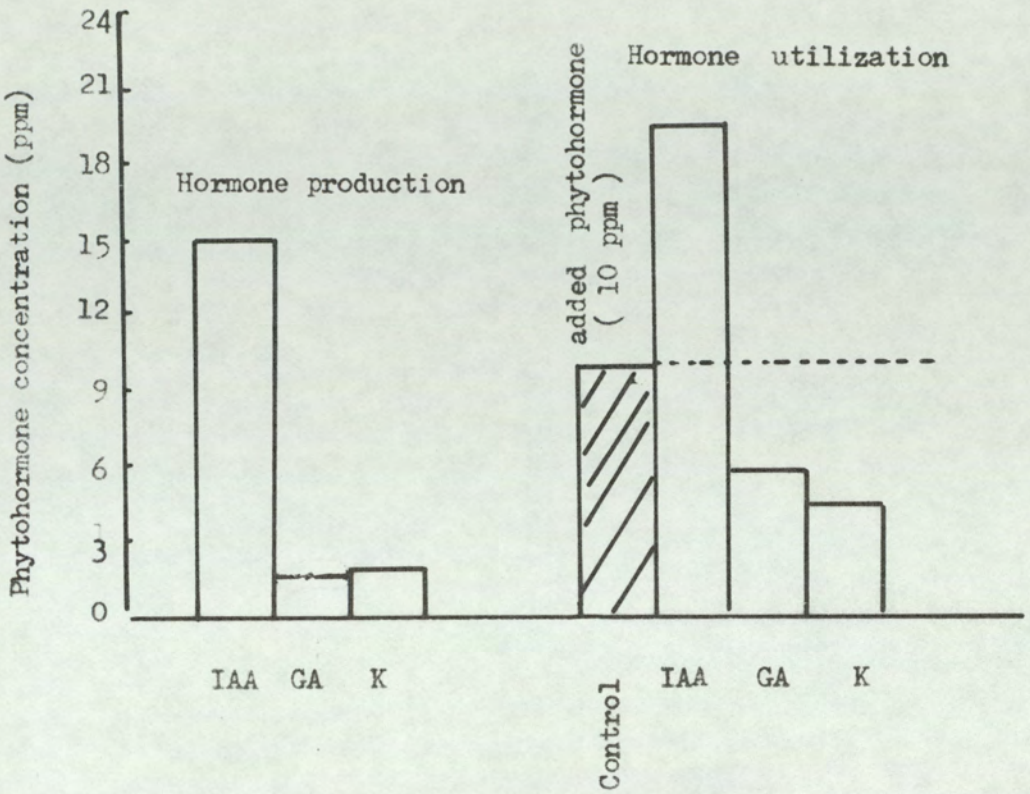
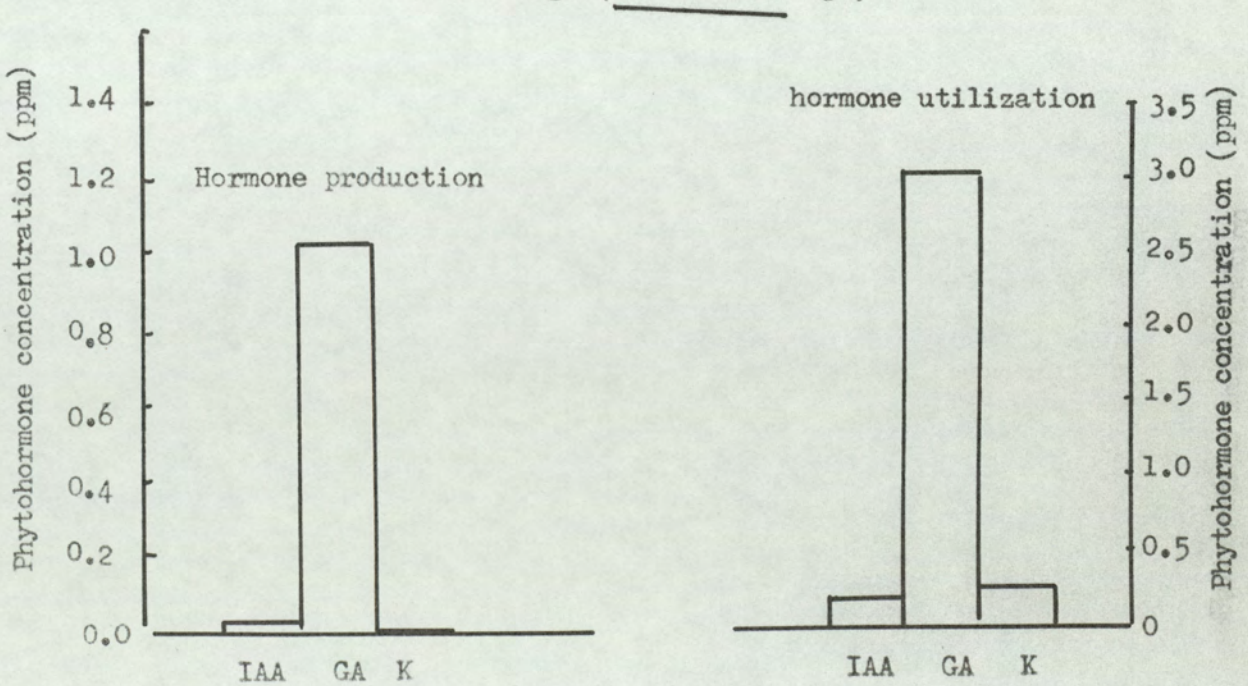


Figure 3.48 :Phytohormone production and utilization by isolated fungus(Penicillium sp.) .



flasks from each treatment were harvested and prepared for hormone analysis (section 2.7., 2.8., 2.9).

Similarly, the isolated fungus was grown in submerged culture (section 2.6). Phytohormones (IAA, GA_3, K) were also added to culture flasks at a concentration of 10ppm. After 7 days of incubation, three flasks from each treatment were harvested and prepared for hormone analysis (section 2.7., 2.8., 2.9). The harvested mycelium was dried for dry weight determination.

The isolated bacteria was not able to utilize or decompose the added IAA. Moreover, the concentration of IAA in the culture medium was significantly increased over the control. Also, it was apparent that the addition of IAA at a concentration of 10ppm did not affect IAA production by the isolated bacteria. (Appendix 4.7., Fig 3.47).

Also, the isolated bacteria utilized added gibberellin and cytokinin but at low level compared to that utilized by A.bisporus (see section 3.7.3) and the isolated fungus.

In contrast, the isolated fungus significantly utilized added IAA, GA_3 and K (Fig 3.48). This was

also associated with significant increases in the dry weight of the harvested mycelium (Appendix 4.8).

3.12 Conclusions

The pasteurized compost and casing soil contained an appreciable amount of auxin, gibberellin and cytokinin. The compost contained high concentrations of both auxin and gibberellin, but low concentration of cytokinin. In contrast, the casing soil contained a high concentration of cytokinin but low concentration of both auxin and gibberellin. This indicates that phytohormones are not limiting factors for the growth of A.bisporus. Results from experiments on hormone production by A.bisporus and two selected micro-organisms in artificial culture, also supported this concept.

There was an almost consistent relationship between the concentrations of phytohormones and the population of bacteria and fungi in substrates. Exception was found with the gibberellin in the casing soil, where the fluctuations in its concentrations were inversely related to the population of bacteria and fungi.

In general, the fluctuation in levels of phytohormones was related to those of the population of microorganisms (fungi, bacteria and others) in artificial culture.

SECTION 4

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

As a result of this study it may be concluded that phytohormones affect the growth and development processes in the cultivated mushroom Agaricus bisporus . In addition, it has been shown that the three phytohormones studied, auxin, gibberellin and cytokinin, are a part of the normal substrate environment and therefore contribute to normal growth.

It has been shown that mycelial growth may be stimulated or inhibited by natural and synthetic auxins in both semi-solid and liquid culture. All of the tested auxins inhibited growth at high concentrations, but promoted growth at lower concentrations. Extremely low concentrations did not affect growth. Generally, the optimum concentration for growth promotion was observed within the range 10^{-6} to 10^{-7}M. In this respect concentration effects on A.bisporus are comparable to those obtained with higher plants (Audus, 1972 a).

Not only the extent of growth was affected by phytohormones, but also some gross morphological characteristics were influenced. The shape, density and frequency of sectoring of the colony on semi-solid media were particularly affected. Thus it was found that high concentrations of auxin (10^{-3} to 10^{-4}M) were

always associated with increasingly dense colonies of an irregular shape. This may be due to a toxicity of auxin at high concentrations in the initial stages of growth which was partially removed by the activity of mycelium over protracted incubation times.

Similarly, in liquid culture the effectiveness of the promoting concentrations of auxin (10^{-6} - 10^{-7} M) decreased as the period of incubation was increased; for example, growth rate in liquid culture decreased after four weeks of incubation (section 3.2). This suggests the utilization of auxin by A.bisporus during a protracted incubation time.

In general, gibberellin induced the same effects as auxin with a few minor exceptions, however, the effect of cytokinin contrasted sharply with those of auxin and gibberellin. Although, cytokinin increased radial growth at a concentration of 10^{-7}M it was generally less effective in stimulating growth. Its principle effect was to stimulate sectoring frequency and delaying the senescence of colonies on semi-solid media. This may be associated with its stimulation of cell division (see section 3.1.5) an effect which is comparable to that found in higher plants.

The concentrations at which phytohormones induced growth promoting and effects on colony morphology were different to those when phytohormones were supplemented as a combined treatment (section 3.1.4). This suggested an interaction between phytohormones and since the three groups of phytohormones are a part of the total substrate, it may be concluded that in the methods of commercial culture, this interaction is more relevant than single effects. It is suggested therefore, that vegetative growth is influenced by a delicate balance at which normal growth occurs. Any disturbance to this equilibrium may lead to abnormal growth, inhibition of radial growth or increased sectoring, conditions frequently encountered in commercial methods.

These studies also confirmed observations reported by Fraser (1955) that IAA stimulated mycelial growth in liquid culture at given concentrations which inhibited growth on semi-solid media. It is suggested that views expressed by Leopold (1955 c) that breakdown of auxin in solution is more rapid than in semi-solid media is relevant to these findings.

Studies on cell elongation and cell division (section 3.1.5) are also indicative that phytohormone effects on vegetative growth in A.bisporus have some

features in common with their effects on cells of higher plants.

These studies have also shown that the reproductive stage of A.bisporus is also affected by phytohormones. Applying phytohormones at high concentration (10^{-4}M) to the compost and casing soil reduced yield, dry weight and number of primordia and sporophores. Increased yield were detected at lower concentrations (10^{-6}M) of GA_3, NAA and 2,4-D (section 3.5).

There was a consistent inverse correlation between dry weight of sporophores and the concentration of phytohormones. This suggests that phytohormones enhanced water uptake, an effect known to be the same in green plants (Hackett & Thimann, 1952; Cleland, 1967; Masuda, 1969; and Onder, 1974).

Phytohormones at some concentrations tested increased stipe length and cap diameter. Since the development of the sporophore is dependent on stipe elongation and pileus expansion (Gruen, 1963; Hagimoto, & Konishi, 1959) phytohormones may, therefore, be important in the development of the sporophore.

A similarity with green plants was also shown when protein content of sporophores fluctuated with hormone application (Davies et al, 1968; Thimann et al, 1957

Hackett et al, 1952; and Musda et al, 1969). It may be suggested, therefore, that phytohormones action in A.bisporus may be associated with protein synthesis but the results obtained are not conclusive and require further investigation.

New information has been revealed on the interesting phase of development concerned with the switch from vegetative to reproductive growth. Primordia formation was shown to be influenced by phytohormones applications at certain concentrations. When applied singly, but especially, when in combination. Since primordia formation only occurs on a malt extract medium (Hayes personal communication) suggested that this medium supplied phytohormones. All three groups of phytohormones were found in malt extract (see also Skoog 1972) and may account for the spontaneous formation of primordia on this medium. Similarly, the action of bacteria on primordia formation in Petri plates may be due to phytohormone effects.

It is clear that phytohormones influence many different facets of growth and development in A.bisporus. Phytohormones were extracted from mycelium, fruit bodies. Levels decreased as the age of sporophore increased, but the highest levels were found at the primordium form-

ation stage, thus again implicating phytohormones with the transition from vegetative to reproductive growth.

The distribution pattern of different phytohormones in different parts of the sporophore also suggest a relationship with the development processes. For example, stipe and pilei contain high levels of auxin and gibberellin but low levels of cytokinin (section 3.8.2) compared to gill tissue. Since growth of the stipe and pileus at the cup stage depends on cell elongation, the high concentrations in stipe and pilei suggest a relationship with the requirement for cell elongation at these sites.

Conclusive evidence that the mycelium of A.bisporus utilizes directly phytohormones was provided in section 3.7.3 in addition to its ability to synthesise and secret phytohormones into a synthetic medium. Moreover, the complexity of the phytohormone balance in the substrate environment of A.bisporus was demonstrated by the role of other micro-organisms in the substrate on the phytohormone balance. A dominant bacteria isolate was more effective as a phytohormone producer, while a dominant Penicillium sp was more effective as a phytohormone consumer than a producer.

High concentrations of phytohormones significantly stimulated growth of the Penicillium isolate. Thus it can be seen that a possible explanation of the occurrence of competitor fungi in commercial methods may be due to the concentrations of phytohormones favouring competitors such as Penicillium at the expense of A.bisporus. This effect was noted when phytohormones were applied at high concentrations to the commercial system. Few days after hormones application, the compost and casing soil were completely colonized by a range of fungi including Penicillium sps; Aspergillus sps. and Trichoderma sps.

The process of mushroom culture may be seen as a complex process, in which phytohormones levels exert significant effects all of which in different ways are relevant to the economics of cultivation. Levels of phytohormones fluctuate throughout the various stages of growth (section 3.9) and similarly levels of associated bacteria and fungi fluctuate throughout the life cycle. Superimposed on these patterns is the influence of A.bisporus activity which may produce or utilize phytohormones. In the mixed culture of A. bisporus cultivation, an inherently variable process, control of phytohormones levels may lead to increased

control over growth and the production of fruits. Present techniques of culture do not appear amenable to such control and at such minute levels of activity.

APPENDICES

APPENDIX 1.1 Effect of IAA on mycelial growth of A.bisporus on semi-solid synthetic medium.

Concentration (M)	RD	SE	Period of incubation
0	25	2.3	
10^{-3}	-	-	
10^{-4}	*16	3.1	TWO WEEKS
10^{-5}	24	1.5	
10^{-6}	26	1.5	
10^{-7}	27	1.7	
10^{-8}	26	2.3	
0	60.1	1.7	
10^{-3}	**15.1	1.6	
10^{-4}	**27	2.0	FOUR WEEKS
10^{-5}	58.3	1.4	
10^{-6}	*65	1.36	
10^{-7}	**66	1.5	
10^{-8}	60	2.4	

RD = radial growth of colony (mm), - = no growth.

SE = standard error.

* = t-value is significant at p = 0.1

** = t-value is significant at p = 0.05

Note: The results given in this table are the mean of six replicates.

APPENDIX 1.2 Effect of NAA on mycelial growth of A.bisporus on semi-solid synthetic medium.

Concentration (M)	RD	SE	Period of incubation
0	22	1.3	
10^{-3}	-	-	
10^{-4}	19	3.2	
10^{-5}	25	2.1	TWO WEEKS
10^{-6}	*26	1.1	
10^{-7}	**28	1.2	
10^{-8}	23	0.9	
0	50	1.2	
10^{-3}	-	-	
10^{-4}	*45	1.6	
10^{-5}	55	3.0	FOUR WEEKS
10^{-6}	***63	1.4	
10^{-7}	****70	1.5	
10^{-8}	***62	3.0	

RD = radial growth of colony (mm), - = no growth.

SE = standard error.

* = t-value is significant at p = 0.1

** = t-value is significant at p = 0.025

*** = t-value is significant at p = 0.01

**** = t-value is significant at p = 0.0005

Note: The results given in this table are the mean of six replicates.

APPENDIX 1.3 Effect of 2,4-D on mycelial growth of A.bisporus on semi-solid synthetic medium.

Concentration (M)	RD	SE	Period of incubation
0	26.8	2.0	
10^{-3}	*10.9	2.5	
10^{-4}	22.9	1.8	
10^{-5}	28.5	2.0	TWO WEEKS
10^{-6}	27.7	1.8	
10^{-7}	28	1.5	
10^{-8}	29.1	2.2	
0	70.9	3.0	
10^{-3}	*19.5	2.3	
10^{-4}	**63.23	1.5	
10^{-5}	72.4	2.0	FOUR WEEKS
10^{-6}	72.45	1.6	
10^{-7}	75.59	1.4	
10^{-8}	72.91	3.0	

RD = radial growth of colony (mm).

SE = standard error.

* = t-value is significant at p = 0.005

** = t-value is significant at p = 0.1

Note: The results given in this table are the mean of six replicates.

APPENDIX 1.4 Effect of GA_3 on mycelial growth of A.bisporus on semi-solid synthetic medium.

Concentration (M)	RD	SE	Period of incubation
0	27	4.0	
10^{-4}	20	5.0	
10^{-5}	26	3.0	TWO WEEKS
10^{-6}	30	2.0	
10^{-7}	32	2.1	
10^{-8}	30	1.5	
10^{-9}	30	1.7	
0	62	3.1	
10^{-4}	*51	3.5	
10^{-5}	63	2.1	FOUR WEEKS
10^{-6}	*70	2.2	
10^{-7}	**75	1.7	
10^{-8}	*70.1	1.9	
10^{-9}	67	1.5	

RD = radial growth of colony (mm).

SE = standard error.

* = t-value is significant at p = 0.1

** = t-value is significant at p = 0.01.

Note: The results given in this table are the mean of six replicates.

APPENDIX 1.5 Effect of kinetin on mycelial growth of *A.bisporus* on semi-solid synthetic medium.

Concentration (M)	RD	SE	Period of incubation
0	27	3.3	
10^{-4}	*20	2.0	
10^{-5}	30	1.7	
10^{-6}	30	2.0	TWO WEEKS
10^{-7}	35	3.0	
10^{-8}	34	3.1	
10^{-9}	32	2.5	
0	67	3.5	
10^{-4}	64	3.0	
10^{-5}	72	2.0	
10^{-6}	73	2.5	FOUR WEEKS
10^{-7}	*78	3.2	
10^{-8}	*78	3.5	
10^{-9}	73	1.8	

RD = radial growth of colony (mm)

SE = standard error

* = t-value is significant at p = 0.1.

Note: The results given in this table are the mean of six replicates

APPENDIX 1.6 Influence of combination of IAA & GA_3 on mycelial growth of A.bisporus on semi-solid medium:

Treatment	RD	SE	CSH	CD	SFR	PIN
Control	25	2.0	+	+	+	
10^{-4}M IAA	*10	1.7	+++	++	+	
10^{-4}M GA_3	20	3.7	++	+	++	
10^{-4}M IAA & 10^{-4}M GA_3	-	-	-	-	-	
10^{-5}M IAA	25	2.5	+	+	++	
10^{-5}M GA_3	25	4.1	+	+	+++	
10^{-5}M IAA & 10^{-5}M GA_3	*35	1.7	+	+	++++	
10^{-4}M IAA & 10^{-5}M GA_3	**11	4.0	+++	++	+	
10^{-5}M IAA & 10^{-4}M GA_3	**15	3.5	+++	++	+	2-week
10^{-6}M IAA	27	4.1	+	+	+++	
10^{-6}M GA_3	32	2.0	+	+	+++	
10^{-6}M IAA & 10^{-6}M GA_3	30	3.4	+	+	+++	
10^{-6}M IAA & 10^{-4}M GA_3	**16	2.7	++	++	++	
10^{-6}M IAA & 10^{-5}M GA_3	*17	3.2	++	++	+	
10^{-5}M IAA & 10^{-6}M GA_3	24	5.1	+	+	+	
10^{-4}M IAA & 10^{-6}M GA_3	**15	1.9	+++	+	-	

Appendix 1.6 continued..

Treatment	RD	SE	CSH	CD	SFR	PIN
Control	58.25	1.9	+	++	+++	
10^{-4}M IAA	*23.25	1.8	++	+	++	
10^{-4}M GA_3	50.16	5.2	++	+	++	
10^{-4}M IAA & 10^{-4}M GA_3	*17.1	1.29	+++	++	+	
10^{-5}M IAA	61.2	3.19	+	+	+++	
10^{-5}M GA_3	59.9	6.11	+	+	++++	
10^{-5}M IAA & 10^{-5}M GA_3	**67.4	1.9	+	+	++++	
10^{-4}M IAA & 10^{-5}M GA_3	*26.8	6.0	+++	++	+	4-week
10^{-5}M IAA & 10^{-4}M GA_3	*42.7	4.0	++	+	++	
10^{-6}M IAA	60.08	5.12	+	+	+++	
10^{-6}M GA_3	**68.7	2.6	+	++	+++	
10^{-6}M IAA & 10^{-6}M GA_3	59.58	4.9	+	+	++++	
10^{-6}M IAA & 10^{-4}M GA_3	**43.5	3.74	++	++	++	
10^{-6}M IAA & 10^{-5}M GA_3	**47.5	4.1	++	++	++	
10^{-5}M IAA & 10^{-6}M GA_3	54.1	6.76	++	+	++	
10^{-4}M IAA & 10^{-6}M GA_3	*30	2.9	++	+	+	

Appendix 1.6 continued .....

PIN = period of incubation

RD = radial growth of colony (mm), - = no growth

SE = standard error

CSH = colony shape + = regular; ++ = slight irregular; +++ = irregular.

CD = density of colony + = normal density; ++ = moderately dense colony

SFR = sectoring frequency + = rare; ++ = few; +++ = many ++++ = intense; - = no sectoring

Note: The results given in this table are the mean of six replicates.

* = t-value is significant at p = 0.01

** = t-value is significant at p = 0.05

APPENDIX 1.7 Influence of combination of IAA & K on mycelial growth of A.bisporus on semi-solid synthetic medium.

Treatment	RD	SE	CSH	CD	SFR	PIN
Control	35	2.5	+	+	+	
10^{-4}M IAA	30	3.1	++	++	+	
10^{-4}M K	34	1.8	++	++	++	
10^{-4}M IAA & 10^{-4}M K	*29	2.5	+++	++	+	
10^{-5}M IAA	36	2.5	+	+	++	
10^{-5}M K	37	1.7	+	+	+++	
10^{-5}M IAA & 10^{-5}M K	37	1.7	+	+	++	
10^{-5}M IAA & 10^{-4}M K	30	3.1	++	+	++	
10^{-4}M IAA & 10^{-5}M K	35	1.8	+	+	+	
10^{-6}M IAA	37	2.5	++	+	++	
10^{-6}M K	35	2.1	+	+	++	two-week
10^{-6}M IAA & 10^{-6}M K	39	1.8	+	++	+++	
10^{-6}M IAA & 10^{-4}M K	*27	2.5	++	+	+	
10^{-6}M IAA & 10^{-5}M K	36	1.8	+	+	++	
10^{-4}M IAA & 10^{-6}M K	*29	1.9	++	+	+	
10^{-5}M IAA & 10^{-6}M K	37	1.8	+	+	++	
10^{-7}M IAA	40	2.6	+	+	++	
10^{-7}M K	41	3.1	+	+	+++	

Appendix 1.7 continued ....

Treatment	RD	SE	CSH	CD	SFR	PIN
10^{-7}M IAA & 10^{-7}M K	**43	1.3	+	+	+++	
10^{-7}M IAA & 10^{-4}M K	36	1.7	+	+	++	
10^{-7}M IAA & 10^{-5}M K	34	3.0	+	+	+++	
10^{-7}M IAA & 10^{-6}M K	37	2.2	+	+	+++	
10^{-4}M IAA & 10^{-7}M K	31	3.1	+	+	++	
10^{-5}M IAA & 10^{-7}M K	36	3.6	+	+	+++	
10^{-6}M IAA & 10^{-7}M K	*41	1.5	+	+	+++	
Control	70	3.2	+	+	+	
10^{-4}M IAA	67	3.2	+	+	++	
10^{-4}M K	69.1	1.9	++	+	+++	
10^{-4}M IAA & 10^{-4}M K	64	2.7	++	++	+++	
10^{-5}M IAA	73	2.6	+	+	++	4-week
10^{-5}M K	74	1.5	+	+	+++	
10^{-5}M IAA & 10^{-5}M K	72	1.4	+	+	++	
10^{-5}M IAA & 10^{-4}M K	70.1	3.5	++	+	+++	

Appendix 1.7 continued ....

Treatment	RD	SE	CSH	CD	SFR	PIN
10^{-4}M IAA & 10^{-5}M K	70.5	1.6	+	+	++	
10^{-6}M IAA	74	4.0	+	+	++	
10^{-6}M K	73.5	2.5	+	+	+++	
10^{-6}M IAA & 10^{-6}M K	*78.5	1.9	+	+	++++	4-week
10^{-6}M IAA & 10^{-4}M K	67	2.7	+	+	++	
10^{-6}M IAA & 10^{-5}M K	71	1.9	+	+	+++	
10^{-4}M IAA & 10^{-6}M K	67	1.4	++	++	++++	
10^{-5}M IAA & 10^{-6}M K	75	2.0	+	+	++++	
10^{-7}M IAA	*79	2.5	+	+	++	
10^{-7}M K	*81.4	3.5	+	+	++++	
10^{-7}M IAA & 10^{-7}M K	***82	1.5	+	+	++++	
10^{-7}M IAA & 10^{-4}M K	74.2	1.5	+	+	+++	
10^{-7}M IAA & 10^{-5}M K	70.5	2.5	+	+	++++	
10^{-7}M IAA & 10^{-6}M K	73	2.3	+	+	++++	

Appendix 1.7 continued ...

Treatment	RD	SE	CSH	CD	SFR	PIN
10^{-4}M IAA & 10^{-7}M K	69	3.2	+	+	++	
10^{-5}M IAA & 10^{-7}M K	74	3.5	+	+	+++	
10^{-6}M IAA & 10^{-7}M K	**80	1.9	+	+	++++	

PIN = period of incubation.

RD = radial growth of colony (mm), mean of six replicates.

SE = standard error.

CSH = shape of colony. + = regular; ++ = slight irregular; +++ = irregular

CD = density of colony. + = normal density; ++ moderately dense colony

SFR = sectoring frequency. + = rare; ++ = few; +++ = many; ++++ = intense

* = t-value is significant at p = 0.1

** = t-value is significant at p = 0.05

*** = t-value is significant at p = 0.025

APPENDIX 1.8 Effect of combination of IAA, GA_3 and K on mycelial growth of A.bisporus on semi-solid synthetic medium.

Concentration (M)	RD	SE	CSH	CD	SFR	PIN
0	36	3.2	+	+	+	
10^{-4}IAA + 6 x 10^{-5} GA + 4 x 10^{-4}K	*19	4.5	+++	++	-	
10^{-4}IAA + 6 x 10^{-5} GA + 5 x 10^{-5}K	32	3.5	+	+	++++	
10^{-4}IAA + 6 x 10^{-5} GA + 5 x 10^{-6}K	28	4.0	+	+	++	
3 x 10^{-5} IAA + 4 x 10^{-4}K + 3 x 10^{-5} GA	***26	5.0	+	+	++++	2 weeks
6 x 10^{-5} IAA + 5 x 10^{-5}K + 3 x 10^{-5} GA	30	2.5	+	+	++	
6 x 10^{-5} IAA + 5 x 10^{-6}K + 3 x 10^{-5}GA	***27	4.0	++	++	++++	
10^{-5} IAA + 4 x 10^{-4}K + 3 x 10^{-6}GA	31	4.0	+	+	++	
10^{-5} IAA + 5 x 10^{-5}K + 3 x 10^{-6}GA	***26	2.6	++	++	++	
10^{-5} IAA + 5 x 10^{-6}K + 3 x 10^{-6}GA	42	2.5	+	++	++++	

Appendix 1.8 continued ....

Concentration (M)	RD	SE	CSH	CD	SFR	PIN
0	70	4.5	+	+	+	
10^{-4} IAA + 6×10^{-5} GA + 4×10^{-4} K	**45	6.0	++	++	++	
10^{-4} IAA + 6×10^{-5} GA + 5×10^{-5} K	***65	6.0	+	+	++++	
10^{-4} IAA + 6×10^{-5} GA + 5×10^{-6} K	57	6.2	+	+	+++	
3×10^{-5} IAA + 4×10^{-4} K + 3×10^{-5} GA	**52	3.2	+	+	++++	4 weeks
6×10^{-5} IAA + 5×10^{-5} K + 3×10^{-5} GA	***60	3.0	+	+	++++	
6×10^{-5} IAA + 5×10^{-6} K + 3×10^{-5} GA	***56	4.0	++	++	++++	
10^{-5} IAA + 4×10^{-4} K + 3×10^{-6} GA	***60	3.0	+	++	++++	
10^{-5} IAA + 5×10^{-4} K + 3×10^{-6} GA	***57	3.6	+	+	+++	
10^{-5} IAA + 5×10^{-6} K + 3×10^{-6} GA	***80	3.0	+	++	+++	

Appendix 1.8 continued

RD = radial growth (mm) mean of six replicates.

SE = standard error.

CSH = shape of colony. + = regular; ++ = slight irregular; +++ = irregular

CD = density of colony. + = normal density; ++ = moderately dense colony.

SFR = sectoring frequency. + = rare; ++ = few; +++ = many; ++++ = intense

PIN = period of incubation.

* = t-value is significant at p = 0.05.

** = t-value is significant at p = 0.025.

*** = t-value is significant at p = 0.1.

APPENDIX 1.9 Effect of phytohormones on the cell length of A.bisporus on semi-solid synthetic medium

Concentration (M)	cell length (μ)	standard error
0	61.2	2.27
10^{-7} IAA	*73.07	2.87
10^{-6} IAA	63.63	1.7
10^{-5} IAA	**70.49	2.13
6×10^{-5} IAA	*74.28	2.4
4×10^{-8} GA_3	*76.26	2.9
3×10^{-7} GA_3	60.31	2.14
3×10^{-6} GA_3	57.73	2.13
3×10^{-5} GA_3	58.6	1.35
5×10^{-8} K	***54.61	1.89
5×10^{-7} K	*48.31	1.4
5×10^{-6} K	60.78	2.44
5×10^{-5} K	56.17	2.60

Note: The results given in this table are the mean of 40 replicates.

* = t-value is significant at p = 0.01

** = t-value is significant at p = 0.05

*** = t-value is significant at p = 0.1

APPENDIX 1.10 Effect of IAA on mycelial growth of *A.bisporus* in liquid culture.

Concentration (M)	pH	SE	DW	SE	PIN
0	5.77	0.093	0.056	0.003	
10^{-4}M	5.75	0.06	**0.047	0.002	
10^{-5}M	*6.05	0.028	0.055	0.02	2-week
10^{-6}M	5.85	0.064	0.05	0.003	
10^{-7}M	**6.125	0.06	**0.077	0.002	
0	6.44	0.15	0.061	0.002	
10^{-4}M	6.46	0.156	**0.086	0.002	
10^{-5}M	6.68	0.058	**0.099	0.001	4-week
10^{-6}M	***6.0	0.194	**0.084	0.002	
10^{-7}M	**6.88	0.08	**0.1274	0.01	

DW = dry weight of mycelium (g/50 ml medium).

SE = standard error.

PIN = period of incubation.

* = t-value is significant at p = 0.025.

** = t-value is significant at p = 0.05.

*** = t-value is significant at p = 0.1.

Note: The results given in this table are the mean of five replicates.

APPENDIX 1.11 Influence of NAA on Mycelial growth of A.bisporus in liquid culture

Treatment	pH	SE	Dry Weight (g)	SE	Period of Incubation
0	5	0.3	0.030	0.004	
10^{-4}M	5.15	0.2	0.033	0.002	
10^{-5}M	5.02	0.3	*0.037	0.001	
10^{-6}M	5.18	0.1	**0.043	0.001	2-week
10^{-7}M	5.24	0.25	0.033	0.002	
10^{-8}M	5.45	0.4	0.0385	0.005	
0	6.08	0.1	0.061	0.002	
10^{-4}M	6.1	0.2	*0.067	0.003	
10^{-5}M	6.03	0.2	***0.0751	0.002	4-week
10^{-6}M	6.3	0.4	***0.085	0.001	
10^{-7}M	6.5	0.34	***0.07	0.002	
10^{-8}M	6.6	0.5	***0.081	0.006	

SE = standard error.

Note: The results given in this table are the mean of five replicates.

* = significant at p = 0.125

** = significant at p = 0.025

*** = significant at p = 0.05

APPENDIX 1.12 Effect of 2,4-D on Mycelial growth of A.bisporus in liquid culture

Concentration	pH	SE	Dry Weight (g)	SE	Period of Incubation
0	6.0	0.32	0.036	0.002	
10^{-4}M	*6.5	0.05	0.036	0.005	
10^{-5}M	6.1	0.05	**0.056	0.002	2 weeks
10^{-6}M	5.9	0.11	**0.06	0.002	
0	6.5	0.1	0.073	0.004	
10^{-4}M	6.52	0.18	0.075	0.004	
10^{-5}M	*6.75	0.1	**0.097	0.002	4 weeks
10^{-6}M	6.5	0.25	0.08	0.002	

SE = standard error

Note: The results given in this table are the mean of five replicates

* = significant at p = 0.125

** = significant at p = 0.05

APPENDIX 1.13 Influence of Gibberellic acid on Mycelial growth of A.bisporus in liquid culture.

Concentration (M)	pH	SE	Dry Weight (g)	SE	Period of Incubation
0	6.1	0.38	0.015	0.002	
10^{-4}M	*5.57	0.047	0.0175	0.002	
10^{-5}M	5.57	0.07	0.0175	0.0025	2 weeks
10^{-6}M	5.65	0.086	*0.025	0.003	
0	6.65	0.155	0.045	0.002	
10^{-4}M	*6.22	0.188	0.042	0.003	
10^{-5}M	6.3	0.17	*0.056	0.003	4 weeks
10^{-6}M	6.77	0.206	*0.062	0.004	

SE = standard error

Note: The results given in this table are the mean of five replicates.

* = significant at p = 0.125

APPENDIX 1.14 Effect of Kinetin on Mycelial growth of A.bisporus in liquid culture.

Concentration (M)	pH	SE	Dry Weight (g)	SE	Period of Incubation
0	5.7	0.3	0.035	0.003	
10^{-4}M	5.2	0.4	**0.025	0.003	
10^{-5}M	5.5	0.2	0.035	0.004	2 weeks
10^{-6}M	5.3	0.5	**0.027	0.003	
10^{-7}M	5.9	0.3	**0.045	0.003	
0	6.9	0.6	0.0763	0.003	
10^{-4}M	6.5	0.5	*0.0411	0.003	
10^{-5}M	6.53	0.15	0.0703	0.002	4 weeks
10^{-6}M	6.9	0.155	0.079	0.002	
10^{-7}M	7.07	0.12	**0.0846	0.003	

SE = standard error.

Note: The results given in this table are the mean of five replicates.

* = significant at p = 0.01

** = significant at p = 0.1

APPENDIX 1.15 Effect of combination of different hormones on A.bisporus growth in liquid culture

Concentration (M)	pH	SE	DW	SE	PIN
0	5.7	0.1	0.03	0.002	
10^{-6} IAA + 3 x 10^{-6}GA	5.95	0.3	0.032	0.002	
10^{-6} IAA + 5 x 10^{-6}K	5.9	0.2	0.0028	0.003	
3 x 10^{-6}GA + 5 x 10^{-6}K	5.8	0.1	0.017	0.004	
10^{-6} IAA + 5 x 10^{-7}K	5.9	0.15	0.023	0.003	
10^{-6} IAA + 3 x 10^{-7}GA	5.9	0.2	0.025	0.005	2 weeks
5 x 10^{-7} K + 3 x 10^{-7}GA	5.8	0.3	0.22	0.003	
10^{-7} IAA + 5 x 10^{-8}K	6.0	0.17	0.023	0.004	
10^{-7} IAA + 4 x 10^{-8}GA	5.9	0.25	0.03	0.003	
4 x 10^{-8}GA + 5 x 10^{-8}K	6.0	0.3	0.027	0.004	
0	6.28	0.17	0.059	0.003	
10^{-6} IAA + 3 x 10^{-6}GA	*6.7	0.11	0.06	0.001	
10^{-6} IAA + 5 x 10^{-6}K	6.66	0.3	0.057	0.003	
3 x 10^{-6}GA + 5 x 10^{-6}K	6.4	0.2	*0.039	0.003	

APPENDIX 1.15 continued....

Concentration (M)	pH	SE	DW	SE	PIN
10^{-6} IAA + 5×10^{-7}K	6.64	0.15	0.048	0.004	
10^{-6} IAA + 3×10^{-7}GA	*6.7	0.13	0.048	0.002	4 weeks
5×10^{-7}K + 3×10^{-7}GA	6.58	0.13	*0.0411	0.002	
10^{-7} IAA + 5×10^{-8}K	*6 9	0.17	*0.0411	0.003	
10^{-7} IAA + 4×10^{-8}GA	*6.7	0.13	0.057	0.004	
4×10^{-8} GA + 5×10^{-8}K	*6.83	0.2	0.0501	0.003	

DW = dry weight of mycelium (g/50ml of medium).

SE = standard error.

PIN = period of incubation.

* = t-value is significant at p = 0.1

Note: The results given in this table are the mean of five replicates.

APPENDIX 1.16 Effect of phytohormones on mycelial growth of A.bisporus on pasteurized compost.

Concentration (M)	LCC	SE	CD
0	73.3	2.58	+
5×10^{-7} K	**65.6	2.71	+++
5×10^{-6} K	*66.6	1.02	+++
10^{-7} IAA	69.8	1.27	++
10^{-6} IAA	68.4	2.65	++
3×10^{-7} GA	72.8	3.3	++
3×10^{-6} GA	**79.2	2.03	++
10^{-6} IAA + 3×10^{-6} GA + 5×10^{-6} K	**79.4	1.627	+++

LCC = length of colonized compost (mm)

SE = standard error

CD = density of colony. + = normal density; ++ = moderately dense colony; +++ = dense colony.

* = t-value is significant at p = 0.05

** = t-value is significant at p = 0.1

APPENDIX 2.1 Effect of IAA on reproductive growth of *A.bisporus* in artificial culture

Concentration (M)	PN	SE	SN	SE	SL	SE	CD	SE	DW	SE	TFW	SE	PC	SE
	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	33	2.9	14	1.75	46.4	1.8	56.5	2.6	9.89	0.65	193	15	25.7	0.69
10^{-4}	*26	2.9	13	1.47	49.9	2.2	56.6	2.4	9.1	0.6	173	10.6	25.8	0.57
10^{-5}	33	3.5	13	0.96	51*	1.9	59.3	2.3	9.4	0.35	192	7.9	*27.3	0.44
10^{-6}	33	3.64	10*	0.85	52.6	1.5	59.6 **	1.9	9.54	0.4	183	9.4	26.19	1.0

PN = primordium number (mean of 8 replicates)

SE = standard error

SN = sporophore number (mean of 8 replicates)

SL = stipe length (mm) (mean of 40 replicates)

CD = cap diameter (mm) (mean of 40 replicates)

DW = dry weight of sporophores (g/100g fresh weight) (mean of 10 replicates)

TFW = total fresh weight (g) (mean of 8 replicates)

PC = protein content (g 100 g^{-1} dry matter) (mean of 10 replicates)

* = t-value is significant at p = 0.12

** = t-value is significant at p = 0.05

APPENDIX 2.2 Effect of NAA on reproductive growth of A.bisporus in artificial culture

Concentration (M)	PN	SE	SN	SE	SL	SE	CD	SE	DW	SE	TFW	SE	PC	SE
0	41	3.9	13	.92	45	1.1	49.3	2.0	8.65	.8	176	9.8	25.7	.55
10^{-4}M	34	3.2	7***	.28	45.5	2.4	*58.5	3.29	8.2	.27	168	24.2	25.2	.34
10^{-5}M	*31	2.8	8**	.9	48.5	1.5	*55.8	2.0	8.83	.9	163	8.1	26.8	.35
10^{-6}M	36	3.5	11	.9	*49.3	1.29	*54.3	1.93	8.67	.55	190	17.3	25.45	.62

PN = primordium number (mean of 8 replicates)

SE = standard error.

SN = sporophore number (mean of 8 replicates)

SL = stipe length (mean of 40 replicates)

CD = cap diameter (mean of 40 replicates)

DW = dry weight (mean of 10 replicates)

TFW = total fresh weight (g) (mean of 8 replicates)

PC = protein content (g 100 g^{-1}) (mean of 10 replicates)

* = t-value is significant at p = 0.125

** = t-value is significant at p = 0.0025

*** = t-value is significant at p = 0.0005

APPENDIX 2.3 Effect of 2,4-D on reproductive growth of A.bisporus in artificial culture

Concentration (M)		PN	SN	CD	SL	DW	TW	PC
0	m	:25	13	45	30	8.7	124	27.5
	se	:0.5	0.56	1.4	1.75	0.65	8.37	0.44
10^{-8}	m	:24	14	47.5	30	8.9	134.7	26.1
	se	:0.4	0.88	1.35	2.6	0.44	12.9	0.5
10^{-7}	m	:26	14	*55	32	8.8	**153.1	30.4*
	se	:0.35	0.51	1.35	1.25	0.44	6.15	0.0005
10^{-6}	m	:23**	16***	52***	35****	9.3	**152.3	30.1**
	se	:0.45	0.55	1.25	1.31	0.56	5.2	0.6
10^{-5}	m	:20*	16***	55*	35**	8.21	128.8	27.56
	se	:0.5	0.6	1.21	1.15	0.43	10.7	0.08

PN = primordium number, mean of 8 replicates

SN = sporophore number, mean of 8 replicates

CD = cap diameter (mm), mean of 30 replicates

SL = stipe length (mm), mean of 30 replicates

DW = dry weight of sporophore (g/100 g fresh weight),mean of 10 replicates

TW = total fresh weight of harvested sporophores (g/600g of compost), mean of 8 replicates

PC = protein content of sporophore (g/100g dry weight), mean of 5 replicates

m = mean, se = standard error

* = t-value is significant at p = 0.0005.

** = t-value is significant at p = 0.05

*** = t-value is significant at p = 0.01

**** = t-value is significant at p = 0.1

APPENDIX 2.4 Effect of GA_3 on reproductive growth of *A.bisporus* in artificial culture

Concentration (M)		PN	SN	SL	CD	DW	TY	PC
0	m :	34	11	43.6	59.1	10.9	122.8	26.4
	se:	3.07	2.9	2.0	3.3	0.44	11.1	0.44
10^{-4}	m :	29	8.0	43.2	55.2	*9.8	99.19	28.18**
	se:	3.8	1.18	1.5	1.9	0.176	11.2	0.2
10^{-5}	m :	36.0	8.0	*39.1	56.2	10.06	108.4	26.1
	se:	4.4	1.5	1.5	2.0	0.17	11.8	0.181
10^{-6}	m :	36.0	8.0	41	56.4	10.08	132	29.92***
	se:	1.27	1.0	1.02	1.65	0.35	12.73	0.141

PN = primordium number, mean of 8 replicates

SN = sporophore number, mean of 8 replicates

SL = stipe length (mm), mean of 30 replicates

CD = cap diameter (mm), mean of 30 replicates

DW = dry weight of sporophore (g/100 g fresh weight), mean of 10 replicates

TY = total yield (g/600 g of compost), mean of 8 replicates

PC = protein content of sporophore (g/100 g dry weight), mean of 10 replicates

* = t-value is significant at p = 0.1

** - t-value is significant at p = 0.025

*** = t-value is significant at p = 0.0005

APPENDIX 2.5 Effect of kinetin on reproductive growth of A.bisporus in artificial culture.

Concentration (M)		PN	SN	SL	CD	DW	TFWY	PC
0	m	:40	5.0	40	50	10.24	120	21.2
	se	:0.5	0.6	0.3	0.43	0.14	4.5	0.20
10^{-6}	m	:43*	4.0	43*	48**	9.76***	122.3	23.5*
	se	:0.43	0.46	0.24	0.35	0.25	6.16	0.123
10^{-5}	m	:37*	3.0***	40	52*	9.2*	108.1	21.02
	se	:0.43	0.31	0.4	0.23	0.06	6.56	0.6
10^{-4}	m	:40	4	41***	53*	9.5*	116	23.1*
	se	:0.45	0.52	0.36	0.37	0.05	3.08	0.18

PN = primordium number, mean of 8 replicates

SN = sporophore number, mean of 8 replicates

SL = stipe length (mm), mean of 40 replicates

CD = cap diameter (mm), mean of 40 replicates

DW = dry weight (g. $100g^{-1}$ fresh weight), mean of 10 replicates

TFW = total fresh weight of harvested sporophores (g/600 g compost), mean of 8 replicates

PC = protein content of sporophore (g/100 g dry weight) mean of 10 replicates

* = t-value is significant at p = 0.005

** = t-value is significant at p = 0.01

*** = t-value is significant at p = 0.1

APPENDIX 2.6 Effect of combination of phytohormones on reproductive growth of A.bisporus in artificial culture.

Concentration (M)		PN	SN	SL	CD	DW	TFWY	PC
0	m :	44	9.0	40	51	9.02	127.5	27.36
	se:	0.45	0.75	0.6	0.7	0.22	9.4	0.34
10^{-7} (IAA+GA+K)	m :	46*	7.0**	39.5	50.5	9.38	109.4	29.2**
	se:	0.51	0.59	0.72	0.52	0.23	8.59	0.90
10^{-6} (IAA+GA+K)	m :	44	7.0**	41	49.3**	8.7	114.82	27.26
	se:	0.45	0.7	0.55	0.71	0.26	10.3	1.0
10^{-5} (IAA+GA+K)	m :	43	8.0	42*	53**	8.30*	120.66	26.7
	se:	0.54	0.544	0.48	0.61	0.17	9.16	0.50

PN = primordium number, mean of 8 replicates

SN = sporophore number, mean of 8 replicates

SL = stipe length (mm), mean of 40 replicates

CD = cap diameter (mm), mean of 40 replicates

DW = dry weight (g/100 g fresh weight), mean of 10 replicates

TFW = total fresh weight of harvested sporophores (g/600g of of compost), mean of 8 replicates

PC = protein content of sporophores (g/100g dry weight), mean of 10 replicates

* = t-value is significant at p = 0.05

** = t-value is significant at p = 0.1

APPENDIX 2.7 Effect of phytohormones on primordium formation on malt extract agar (single-phase method).

Treatment	Primordium number	Standard error
0	8	2.7
10^{-6}M IAA	9	1.9
10^{-5}M IAA	5	2.7
10^{-4}M IAA	11	1.0
10^{-6}M GA_3	8	2.6
10^{-5} M GA_3	16	7.0
10^{-4}M GA_3	6	2.2
10^{-6}M K	8	3.0
10^{-5}M K	8	2.19
10^{-4}M K	*26	5.55
10^{-5}M IAA & 10^{-5}M GA_3	*27	6.5
10^{-5}M IAA & 10^{-5}M K	17	5.7
10^{-5}M IAA & 10^{-5}M GA_3 & 10^{-5}M K	14	3.1

Note: The results are the mean of five replicates.

* = t-value is significant at P = 0.05.

APPENDIX 2.8 Effect of phytohormones on primordium formation on malt extract and water agar (Two-phase method).

Treatment	PNW	SE	PNM	SE	PNT
0 (control)	8	1.8	5	0.88	13
$10^{-6}GA_3$	10	4.4	8	1.78	18
$10^{-5}GA_3$	12	1.6	15	2.8	27
$10^{-4}GA_3$	7	2.1	8	4.3	15
$10^{-6}K$	16	4.9	7	1.78	23
$10^{-5}K$	11	2.4	11	4.16	22
$10^{-4}K$	10	2.0	***37	6.4	27
$10^{-6}IAA$	10	2.19	***14	3.3	24
$10^{-5}IAA$	*13	2.0	11	2.6	24
$10^{-4}IAA$	10	2.6	***13	2.16	23
$10^{-5}GA_3$ & $10^{-5}IAA$	*19	5.3	*31	17.6	50
$10^{-5}IAA$ & $10^{-5}K$	9	2.9	***14	3.57	23
$10^{-5}GA_3$ & $10^{-5}K$ & $10^{-5}IAA$	7	0.92	*32	20.2	40

PNW = primordium number on water agar phase.

SE = standard error.

PNM = primordium number on malt extract phase.

PNT = primordium number on whole plate (total number of primordia in each plate).

* = t-value is significant at p = 0.125

** = t-value is significant at p = 0.01

*** = t-value is significant at p = 0.0025

**** = t-value is significant at p = 0.05

Note: The results given in this table are the mean of five replicates.

APPENDIX 2.9 Hormone analysis of malt extract.

Sample		IAA(ppm)	GA(ppm)	K(ppm)
1		12.246	9.0508	28.673
2		6.0215	14.334	12.329
	Mean	9.133	11.693	20.501
	Standard error	3.11	2.643	8.171

Note: The results given in this table are based on the dry weight of malt extract.

APPENDIX 3.1 Phytophormone production by A.bisporus mycelium.

Period		IAA(ppm)	GA(ppm)	K(ppm)	DW
1st week	M:	0.2	0.534	0.05	0.00275
	SE:	0.035	0.07	0.003	0.0013
2nd week	M:	1.15	0.661	1.086	0.0195
	SE:	0.07	0.1	0.02	0.001
3rd week	M:	0.379	0.005	0.847	0.071
	SE:	0.04	0.005	.042	0.0025
4th week	M:	0.14	0.009	1.913	0.1535
	SE:	0.05	0.002	0.37	0.032

M = mean of three replicates (ppm)

SE = standard error

DW = dry weight of harvested mycelium (g/50 ml medium).

APPENDIX 3.2 Phytohormone utilization by A.bisporus mycelium.

Period of incubation		IAA	DW	GA	DW	K	DW	DWC
1st week	M:	1.75	0.0065	5.5	0.0065	5.51	0.0092	0.0017
	SE:	0.17	0.0004	0.13	0.0002	0.25	0.0002	0.0005
2nd week	M:	1.085	0.072	3.085	0.0275	1.786	0.0145	0.025
	SE:	0.2	0.007	0.21	0.0023	0.21	0.004	0.001
3rd week	M:	0.86	0.13	0.9	0.068	1.05	0.043	0.035
	SE:	0.13	0.0029	0.15	0.004	0.11	0.0001	0.0025
4th week	M:	0.245	0.1423	0.44	0.095	0.65	0.081	0.070
	SE:	0.11	0.0204	0.21	0.003	0.15	0.002	0.004

M = mean of three replicates (ppm).

SE = standard error.

DW = dry weight of mycelium (g/50 ml medium)

DWC = dry weight of mycelium in the control (g/50 ml medium)

APPENDIX 3.3 Phytohormone amount in different stages of sporophore.

Stage of sporophore			IAA	GA_3	K
	Sl = 3-5mm	sample 1	6.97ppm	20.37ppm	19.5ppm
primordium		sample 2	9.6ppm	30.45ppm	21.5ppm
2 days	Cd = 3-6mm	M	8.28ppm	25.41ppm	20.5ppm
		SE	1.315	5.04	1.0
	Sl = 8-11mm	sample 1	0.11ppm	3.32ppm	27.1ppm
Button		sample 2	0.03ppm	3.7ppm	40.3ppm
3 days	Cd = 8-14mm	M	0.07ppm	3.51ppm	33.7ppm
		SE	0.04	0.19	6.6
	Sl = 20-30mm	sample 1	0.11ppm	3.568ppm	21.0ppm
Cup		sample 2	0.01ppm	2.57ppm	15.6ppm
5 days	Cd = 25-35mm	M	0.06ppm	3.069ppm	18.3ppm
		SE	0.6	0.499	3.38
	Sl = 40-60mm	sample 1	0.02ppm	1.306ppm	2.79ppm
Flat		sample 2	0.01ppm	2.852ppm	3.89ppm
7 days	Cd = 50-65mm	M	0.015ppm	2.079ppm	3.34ppm
		SE	0.005	0.77	0.55

S = sample. Sl = stipe length (mm)

Cd = cap diamater (mm). M = mean of two replicates (ppm/dry weight).

SE = standard error

APPENDIX 3.4 Distribution of phytohormone in different parts of sporophore.

Material		IAA(ppm)	GA(ppm)	K(ppm)
Gills	S1	0.1016	2.66	3.0
	S2	0.15	4.577	3.95
	M	0.1258	3.616	3.475
	SE	0.024	0.9585	0.475
Pileus	S1	0.18	3.131	0.901
	S2	0.165	7.457	0.970
	M	0.172	5.294	0.9355
	SE	0.007	2.16	0.0343
Stipes	S1	0.700	4.44	1.14
	S2	075	6.48	1.55
	M	0.725	5.46	1.345
	SE	0.025	1.02	0.205

S1 = sample number 1. S2 = sample number 2.

M = mean of two replicates (ppm/dry weight).

SE standard error.

APPENDIX 4.1 Phytohormone concentrations in compost throughout the life-cycle of A.bisporus.

DAY		IAA	GA	K
0	M	1.185	1.15	0.37
	SE	0.195	0.2	0.06
18	M	0.0055	1.25	4.48
	SE	0.0052	0.6	2.3
28	M	0.1377	5.99	7.25
	SE	0.015	3.58	5.23
45	M	0.0987	5.09	7.684
	SE	0.001	2.04	5.69
53	M	0.3	4.57	7.15
	SE	0.075	1.45	3.15

M = men of two replicates (ppm/dry weight)
SE = standard error

APPENDIX 4.2 Phytohormone concentrations in casing soil throughout the life-cycle of A.bisporus.

DAY		IAA	E GA	E K
0	M	0.0028	0.46	7.6
	SE	0.0015	0.054	1.1
28	M	0.000737	8.825	2.77
	SE	0.00051	0.577	2.33
45	M	0.645	0.589	10.8
	SE	0.091	0.15	5.8
53	M	0.0875	3.65	7.64
	SE	0.03	1.974	3.25

M = mean of two replicates (ppm/dry weight)
SE = standard error

APPENDIX 4.3 Population of bacteria and fungi in compost.

Intervals	Bacteria	Fungi
	Numbers x 10^9 g^{-1} (dry weight)	Numbers x 10^6 g^{-1} (dry weight)
DAY 0 (prepared compost)	85	13
DAY 18	43	5
DAY 28 (10 days after casing)	80	380
DAY 45 (after 1st flush)	28	3
DAY 53 (after 2nd flush)	6	1

APPENDIX 4.4 Population of bacteria and fungi in casing soil.

Intervals	Bacteria	Fungi
	Numbers x 10^9 g^{-1} (dry weight)	Numbers x 10^7 g^{-1} (dry weight)
DAY 18 (prepared casing)	1	42
DAY 28 (10 days after casing)	260	3
DAY 45 (after 1st flush)	7944	21
DAY 53 (after 2nd flush)	16	3

APPENDIX 4.5 Production of phytohormones by isolated bacterium.

Sample	IAA(ppm)	GA(ppm)	K(ppm)
1	13.117	2.5	0.678
2	17.25	1.01	3.628
3	14.75	0.7	0.95
M	15.2	1.4	1.75
SE	1.35	0.55	0.93

M = mean of three replicates (ppm)
SE = standard error

APPENDIX 4.6 Phytohormone production by isolated fungus.

	IAA	GA	K	DW
M	0.00757	1.02	-	0.216
SE	0.0001	0.44	-	0.006

M = mean of three replicates (ppm).

SE = standard error.

DW = dry weight of harvested mycelium (g/50 ml medium).

APPENDIX 4.7 Utilization of phytohormones by isolated bacteria.

Sample	IAA(ppm)	GA(ppm)	K(ppm)
1	16.716	5.0	1.695
2	21.7	7.5	5.5
3	20.17	4.5	6.0
M	19.52	5.66	4.39
SE	1.475	0.92	1.361

M = mean of 3 replicates of final concentrations (ppm)

SE = standard error.

APPENDIX 4.8 Utilization of phytohormones by isolated fungus.

	IAA	DW	GA	DW	K	DW	DWC
M =	0.19	0.2595	3.02	0.2725	0.241	0.3451	0.216
SE =	0.002	0.028	1.14	0.011	0.5	0.024	0.006

M = mean of three replicates of final concentrations (ppm)

SE = standard error.

DW = dry weight of harvested mycelium (g/50 ml).

DWC = dry weight of harvested mycelium (control), g/50ml medium.

Appendix 5.1 Statistical analysis

1 t-test (Bhattacharyya & Johnson, 1977)

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where $\bar{X}$ = sample mean, S = standard deviation,

n = number of replicates.

The difference between treatments is significant when

calculated t $\geq$ tabulated t

2 Confidence limits (Bhattachayya & Johnson, 1977)

$$\bar{X} + t\sqrt{\frac{S^2}{n}} = \text{upper limit} \qquad \bar{X} - t\sqrt{\frac{S^2}{n}} = \text{lower limit}$$

3 Correlation test and linear relationship (Bhattachayya & Johnson, 1977)

Correlation test

$$r = \frac{\sum XY - \frac{(\sum X)(\sum Y)}{n}}{\sqrt{\left[\sum X^2 - \frac{(\sum X)^2}{n}\right] \cdot \left[\sum Y^2 - \frac{(\sum Y)^2}{n}\right]}}$$

Where X, Y = variables, n = number of samples,

r = correlation coefficient.

There is correlation between the variables when

calculated r $\geq$ tabulated r (at a given level of signifance with n-2 degrees of freedom)

Linear relationship:

The following equation was used to find "best-fitting" lines:

$$Y = a + bX$$

Where Y, X = variables, a = intercept, b = slope.

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