ASPECTS OF THE ROLE OF CYTOKININS IN ADVENTITIOUS ROOT FORMATION.

by

Joslyn Leanda Susan Taylor

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in

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This thesis is dedicated to the memory of my father, Harry Taylor.

DECLARATION

The experimental work described in this thesis was conducted in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of Professor J. van Staden.

These studies were the result of my own investigations, except where the work of others is acknowledged.

Joslyn Leanda Susan Taylor

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ABSTRACT

The initiation and development of adventitious roots in cuttings are highly complex processes, influenced by both endogenous and exogenous factors. These vary from the environmental factors prior to the striking of the cutting, to the anatomical and physiological factors within the stem. Encompassed are the nutrient status, physiological age and degree of differentiation of the tissues, and the balance of endogenous rooting inhibitors and/or promoters (including hormones).

The role of cytokinins in root initiation and development has been perceived as that of an inhibitor. This investigation considered several aspects of the role played by cytokinins in the process of root development.

A qualitative/quantitative analysis of the cytokinin-like activity in stem cuttings of several plants, including both easy- and difficult-to-root species was conducted on a comparative basis. There was no clear correlation between the type / level of cytokinins detected in the cuttings and the relative ease of root formation. Both qualitative and quantitative changes in the compounds exhibiting activity in the soybean callus bioassay were observed over the period of root formation in ***Impatiens*** stem cuttings.

The effects on root formation in cuttings of exogenously applied auxins and cytokinins were investigated. Auxins generally promoted root number and elongation at relatively high concentrations (10^{-4} M), but showed less effect on lateral root initiation and development. At high concentrations, cytokinins strongly inhibited root development, but did promote lateral root growth. In suspension culture, the effect of these hormones differed slightly, with IAA and IBA having no significant effect on root development, but NAA strongly stimulating lateral root initiation. Zeatin (10^{-11} M) significantly increased root length and the number of lateral roots produced.

The effect of treatment of the stem cuttings with potassium permanganate and centrifugation was examined. While both these treatments have been perceived to increase root production in cuttings, no significant improvement in rooting ability following centrifugation (relative to the control) was observed. *Impatiens* cuttings centrifuged in the presence of distilled water showed a significantly reduced rooting ability relative to those centrifuged in the dry state. Treatment with an 8-hour pulse in 0.05% potassium permanganate significantly increased the average root length.

These treatments had an effect on the cytokinin levels and distribution in the stem cuttings. Slightly higher levels of cytokinins were associated with the increase in root number and length in both experiments.

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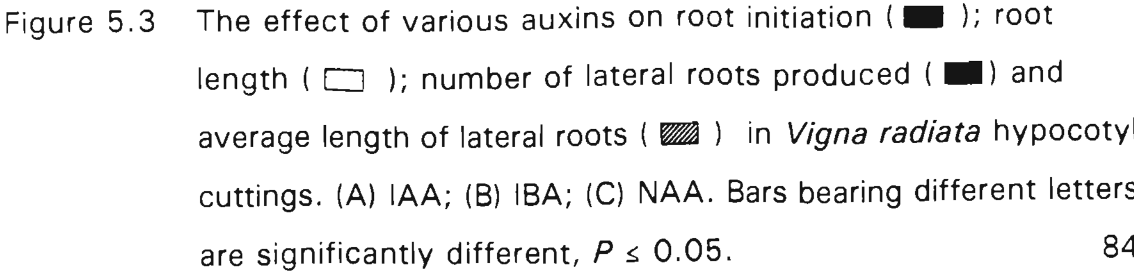

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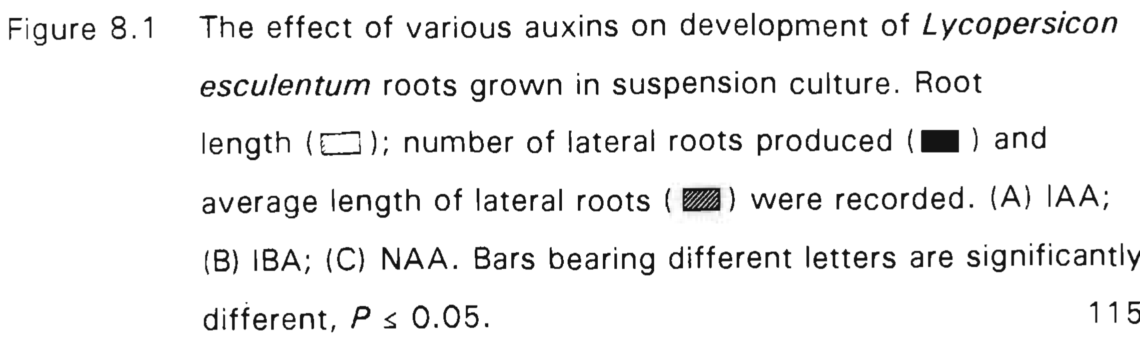

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LIST OF ABBREVIATIONS

BA 6-Benzylaminopurine
DHZ . . . . Dihydrozeatin
DHZ-OG. . . . . Dihydrozeatin-O-glucoside
DHZR Dihydrozeatin-9-riboside
DHZR-OG Dihydrozeatin-9-riboside-O-glucoside
DHZMP . . . . Dihydrozeatin-monophosphate
GA . . . . Gibberellin
IAA. . . . . Indoleacetic acid
IBA . . . . Indolebutyric acid
iP . . . . Isopentenyladenine
iPA . . . . Isopentenyladenosine
iPG . . . . Isopentenyladenine-glucoside
iPMP . . . . Isopentenyladenine-monophosphate
K . . . . Kinetin
NAA . . . . A-Naphthaleneacetic acid
Z . . . . Zeatin
Z-OG . . . . Zeatin-O-glucoside
ZR. . . . . Zeatin riboside
ZR-OG Zeatin-O-glucoside-9-riboside
ZRMP . . . . Zeatin riboside-monophosphate

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The growth and development of plants are regulated by the coordinated interaction of phytohormones and their antagonists, endogenous growth inhibitors. These are active in microquantities (KEFELI, 1978). Agriculture makes extensive use of plant growth regulators. Auxin, particularly, is used as a rooting promoter. This practice is complicated by the complex interactions between two or more classes of growth regulators involved in any type of growth or differentiation process (WAREING, 1978). In many of these processes, for example rooting, cytokinins and auxins are known to interact (CROUCH, 1993), although little is known about the mechanisms of interaction at the metabolic and cellular levels.

1.2 Root formation

Plant roots serve to anchor the plant in a substrate, act in absorption, and as an important source of phytohormones (primarily cytokinins). Roots are involved in the uptake of water and inorganic ions by diffusion through root hairs, or directly through the thin walled epidermis of the root, and in the control of water and inorganic ion distribution to the rest of the plant (TORREY, 1976). In addition, roots may function as reserve storage organs or as a primary means of vegetative reproduction under natural conditions (MAHLSTEDE AND HABER, 1957). Substances synthesized in the root system may exert hormonal control over processes involved in the

maintenance, growth and development of the shoot (CARR AND REID, 1968; TORREY, 1976). The root system in the plant, and the root tip in particular, has been conclusively shown to produce cytokinins (ERIKSEN, 1974; STENLID, 1982; BOURQUIN AND PILET, 1990). Other sites of high meristematic activity (namely the vascular cambium, buds and developing seeds) have been proposed as sites of synthesis (VAN STADEN AND DAVEY, 1979). The remaining plant parts are supplied with cytokinins via the xylem, and possibly the phloem (VAN STADEN AND DAVEY, 1979).

In many respects, root tissues are fundamentally similar to those of the stem, both in structure and function. Xylem and phloem strands are arranged radially in the apical tip of the root. With the development of secondary growth, the arrangement typically becomes continuous with the stem (MAHLSTEDE AND HABER, 1957).

Anatomical processes occurring at the site of root initiation are fundamentally similar for most species and the latter processes in adventitious root formation closely resemble those for lateral root production. In adventitious root formation, the anatomical changes differ only in minor respects, e.g. in the length of vascular connections and whether additional cortical cells are incorporated into the primordium. Precise cell arrangements at the root apex do differ to some extent (LOVELL AND WHITE, 1986).

Lateral and adventitious roots develop from differentiated cells that must re-differentiate to initiate a new root meristem (AESCHBACHER, SCHIEFELBEIN AND BENFEY, 1994; HAND, 1994). The sites of both adventitious and lateral root initiation are usually close to pre-existing vascular tissues (WILSON, 1994). Adventitious roots differ from lateral roots in that they do not originate in normal acropetal sequence from the radicle. This definition encompasses roots that develop on aerial plant parts, underground stems and relatively old roots (ESAU, 1977). In addition, roots that develop as a result of wounding fall into this category. Adventitious root formation is thus evidence of a

regenerative response (BARLOW, 1994).

In lateral root formation, the first steps in the formation of a new root meristem involve changes in the pericycle cells that lie opposite xylem elements (AESCHBACHER, SCHIEFELBEIN AND BENFEY, 1994). Lateral roots originate in the pericycle, a layer of cells immediately exterior to the vascular cylinder. The region of lateral root formation lies behind the root hair zone. In order for a lateral root to form, a region of the pericycle must revert to the meristematic condition. These cells divide to form a growing point, including a root cap, which forces a path mechanically through the endodermis, cortex and epidermis to emerge as a lateral root (MAHLSTEDE AND HABER, 1957). It is not at this time possible to predict where lateral roots will form. Due to the lack of initiation near the root tip of higher plants, it has been suggested that an inhibitor of lateral root formation may diffuse from the primary root apex (AESCHBACHER, SCHIEFELBEIN AND BENFEY, 1994). Despite the many similarities between the processes of initiation of lateral roots and adventitious roots, these processes are not identical (FABIJAN, TAYLOR AND REID, 1981).

Adventitious roots develop spontaneously on intact plants (at nodes of prostrate stems, rhizomes and stolons) or may develop as a wounding response when part of the plant has been deliberately or accidentally severed from the original root system (LOVELL AND WHITE, 1986). Adventitious roots are produced rapidly and profusely in some species under appropriate conditions, but less readily in others. Plants can be grouped according to ease of rooting (LOVELL AND WHITE, 1986).

With respect to the initiation of adventitious roots from stems, there is no consistent tissue of origin. The origin, as determined by the location of meristematic activity, varies with the stage of maturity of the shoot at the time of root formation (MAHLSTEDE AND HABER, 1957), and appears to be related to the time taken for the root to emerge (BATTEN AND GOODWIN,

1978). In general, root initials in young stem material originate in, or close to, the pericycle. As the stem matures, and changes in structure, the site of origin moves progressively inwards from the pericycle to the outer primary phloem parenchyma, to the inner primary phloem parenchyma, to the outer secondary phloem parenchyma and finally to the inner secondary phloem parenchyma. This results in the site of initiation of the primordia being located near the cambium (MAHLSTEDE AND HABER, 1957; BATTEN AND GOODWIN, 1978). Generally, the site of origin is associated with the vascular rays (MAHLSTEDE AND HABER, 1957). As the site moves inwards there is an increase in the time taken for the roots to develop and emerge. Root primordia commonly occur near vascular tissue, possibly since this provides a source of nutrients and hormones (BATTEN AND GOODWIN, 1978).

Preformed adventitious roots develop naturally from definite loci at or near the nodes on the stems of some plants which are still attached to the parent plant, e.g. *Salix* spp. (MAHLSTEDE AND HABER, 1957), poplar (OKORO AND GRACE, 1976), and raspberry (THIMANN, 1977). These are referred to as morphological roots, and do not emerge until a stem piece is severed (MAHLSTEDE AND HABER, 1957). Wound roots, however, develop as a wounding response at any location on the stem, after a cutting is made (HARTMANN AND KESTER, 1983).

Stem tissue is strongly polarised, with cuttings forming shoots at the distal end and roots at the proximal end. This tendency is not affected by changing the position of the cutting with respect to gravity. This polarity is thought to implicate the vascular system as opposed to the basipetal transport of IAA alone (WILSON, 1994). Stem tissue shows the strongest regeneration polarity, followed by roots and lastly leaves, where the new roots and shoots originate at the same position (HARTMANN AND KESTER, 1983).

Cells are said to be "competent" for root formation when they are able to respond directly to an inducing stimulus (e.g. wounding and/or auxin) by the

direct formation of root primordia. Cells that are not competent cannot respond directly to the stimulus, but may become competent indirectly via non-directed cell division. This shift in the development of the cells involves complex interacting changes at the biochemical level as well as at the level of gene expression. Many compounds, including auxins, are involved in this process (HAND, 1994).

Root formation involves a sequence of morphogenetic events with differing requirements (MULLINS, 1972). The process of adventitious root formation can be divided into three stages, namely dedifferentiation, induction and differentiation (DE KLERK, KEPPEL, TER BRUGGE AND MEEKES, 1995). These can be described further as:

1. cellular dedifferentiation followed by meristematic cell initiation (root initial formation);
2. development of the root initials into recognisable root primordia;
3. formation of vascular connections with the conducting tissues of the cutting, and the rupturing of other stem tissue to allow emergence of the new roots (HARTMANN AND KESTER, 1983).

JARVIS (1986) hypothesized that the first phase of the regenerative process in root formation is an inductive or preparatory one, characterized by a lack of cell division and the accumulation of auxin. The second phase is the early initiation phase characterized by cell division and is inhibited by gibberellins (GA). Both these phases would fall within the first stage described by HARTMANN AND KESTER (1983). The root formation process in mung bean cuttings comprises:

1. cell division 0-24 hours;
2. primordia organisation 24-72 hours;
3. emergence of root initials 72-144 hours (WIESMAN AND RIOV, 1994).

These three phases of adventitious root formation may not necessarily be discrete, i.e. it is not clear if all root primordia are induced simultaneously but

develop at different rates, or if induction is sequential (MULLINS, 1972).

The initiation of adventitious roots in cuttings is followed by considerable metabolic activity. New root tissues develop and the roots grow through and out of the surrounding stem tissue to become external functioning roots. Protein synthesis and RNA production have been shown to be indirectly involved in adventitious root development in etiolated stem segments of *Salix tetrasperma* (HARTMANN AND KESTER, 1983).

1.3 Propagation of plants by cuttings

Many plants reproduce naturally by asexual or vegetative processes. This type of reproduction results in the progeny exhibiting exactly the genetic characteristics of the parent from which it was produced. This has enabled the standardisation of many agricultural crops. Vegetative plant propagation is used when there is an inability to produce viable seed; for plants that vary considerably in their genetic makeup; to perpetuate a particular form of plant (often juvenile); to increase the rate of propagation; to develop immunity to pests or an adaption to the habitat; to modify the growth habit (often dwarf) of the plant (MAHLSTEDE AND HABER, 1957).

Cuttings closely follow seed as the most popular methods of plant propagation (WRIGHT, 1973; BARLOW, 1994). Vegetative or clonal propagation is the most important method used for the commercial reproduction of most horticultural crops (DAVIES, DAVIS AND KESTER, 1994). The most important class of propagule utilized in the ornamental plant industry is cuttings, and various fruit and nut crops are propagated in part or exclusively by cuttings (DAVIES, DAVIS AND KESTER, 1994). The vegetative propagation of plants using the technique of cuttings has been in practice for many centuries. Despite this, cuttage is not yet successful enough to meet public and commercial demand (HAISSIG AND DAVIS, 1994). It is estimated

that in the UK alone, 200 million cuttings are taken annually, with as many as 40% failing (BLAKESLEY AND LENTON, 1987).

Adventitious root formation is a prerequisite for the successful clonal regeneration of propagules. Poor rooting continues to be a serious limitation in the commercial asexual propagation of many horticultural crops, especially woody species (DAVIES, DAVIS AND KESTER, 1994). With respect to agronomic crops, adventitious roots are important for those crops that are vegetatively propagated (e.g. sugarcane) and those for which the final yield (fruit/dry matter) is influenced by the presence of adventitious roots (e.g. maize, rice, forage species). The formation of adventitious roots is thus of great significance in the growth and development of agronomic species (KOVAR AND KUCHENBUCH, 1994). Research into the fundamental biology of adventitious rooting is thus of continuing importance.

The propagation of plants by cuttings involves the removal of a vegetative portion of the stem (bulb/rhizome/tuber/corm), root or leaf of the parent plant. When placed under certain favourable conditions these can be induced to form roots and shoots. New plants thus produced are usually identical to the parent (HARTMANN AND KESTER, 1983).

The use of cuttings in propagation brings plants to maturity more quickly than from seeds and gives uniform sized stocks for planting. The rate of root formation is important since rapid rooting minimises cutting exposure to adverse environmental factors, and to the diseases to which unrooted cuttings are prone (HOWARD, 1994). Hormone-treated cuttings generally root more rapidly and have heavier root systems. There is much variation in the rooting response of cuttings from different individuals of the same species and also among varieties within a species. Success in rooting depends largely on the physiological condition of the plant from which cuttings are struck. In addition, differences in the age of the plant, and the position from which the cutting is taken are factors in rooting that must be considered (AVERY AND

JOHNSON, 1947).

It is necessary to understand the internal controls of adventitious rooting in order to comprehend both the fundamental developmental biology of rooting, and to improve rooting for commercial purposes (HAISSIG, DAVIS AND RIEMENSCHNEIDER, 1992). The rooting of vegetatively propagated leafy cuttings involves the complex interaction of many processes (DICK AND DEWAR, 1992). The number of physiological and biochemical processes involved in root formation is so large that the factors that regulate the rooting process have been difficult to identify (RIEMENSCHNEIDER, 1994). The primary endogenous factors controlling the initiation and development of adventitious roots from cuttings include the carbon, water and nutrient status of the cutting as well as hormonal factors (DICK AND DEWAR, 1992). A cutting must contain sufficient food reserves in its tissues to facilitate survival until the formation of new roots and shoots (WRIGHT, 1973). In addition, the effect of the external environment on these endogenous factors both prior to, and subsequent to the detachment from the parent plant, is important. These factors include light intensity, air/soil temperatures and nutrient supply (DICK AND DEWAR, 1992).

1.3.1 Types of cuttings

The type of cutting used in propagation depends on a number of factors including the ease of root or shoot formation, facilities available for propagation and the season of the year. Cuttings are classified first according to the particular plant part used. Four general categories encompass roots, stems, leaves and specialized structures such as tubers and rhizomes. Further classification is based on the maturity of the tissues used and the method of preparation of the cutting. With respect to stem cuttings the categories include softwood cuttings (including both herbaceous and greenwood

cuttings), intermediate or firmwood cuttings and hardwood cuttings (MAHLSTEDE AND HABER, 1957).

Herbaceous cuttings are those taken from soft, succulent seed plants that do not undergo secondary growth. The origin of roots and their rates of growth have been directly correlated with the amount of foliage remaining on the cutting, although too much foliage can result in wilting. Root formation generally occurs relatively quickly (MAHLSTEDE AND HABER, 1957).

Greenwood cuttings are stem cuttings taken from woody plants, prior to lignification while the stems are still relatively soft. These differ from herbaceous cuttings in the maturity of the tissues. Adventitious root formation in these cuttings is a function of both the physiological and anatomical conditions existing in the stem. Factors affecting the success of propagation of these cuttings include pre-existing conditions of food supply; juvenility and hormone balance (MAHLSTEDE AND HABER, 1957).

Intermediate or firmwood cuttings include ripewood, broadleaf (evergreen) and evergreen cuttings. A ripewood cutting is taken later in the season than a softwood cutting. The lower portion of the stem is usually lignified. Leaves are retained. Such cuttings are used in the propagation of species that root over a longer period. This necessitates the inclusion of more mature tissues, since softer tissues are more subject to decay if they remain in the rooting medium with no, or very slow, formation of roots. Broadleaf and evergreen cuttings are taken towards the end of the growing season (MAHLSTEDE AND HABER, 1957).

Unlike deciduous plants, evergreens do not have alternate periods of nutrient accumulation and depletion. Instead, they synthesize and store food throughout the year, the rate varying with temperature and the availability of water and nutrients. The ability of cuttings to root is thus associated with juvenility; the distribution and activity of natural plant auxins; the phase of growth, as determined by season and the anatomical modifications of the

tissues (MAHLSTEDE AND HABER, 1957).

Hardwood cuttings differ from greenwood cuttings in the stage of growth, as determined by the presence or absence of foliage, and the degree of lignification. Hardwood cuttings are taken from deciduous plants that root readily, during the dormant season when the tissues are fully matured and generally lignified along their entire length (MAHLSTEDE AND HABER, 1957). Moderately vigorous, well matured one-year-old wood, containing viable buds, is best selected for cuttings. Typically there is a basal slant cut and a horizontal terminal cut, to remove the tip. Shoots are best collected in early winter after one or two sharp frosts (MAHLSTEDE AND HABER, 1957).

The balance between the carbohydrate and nitrogen content of the reserve food supply in the stem at the time of striking a cutting is important with respect to the ability of the cutting to initiate and develop roots. Stems of dormant deciduous plants with a high starch reserve generally root best (MAHLSTEDE AND HABER, 1957).

1.3.2 Response of plant tissues to wounding

Plants, if not lethally damaged, are able to regenerate the destroyed tissue, and protect against parasitic attack and water loss through a process of wound healing (BARCKHAUSEN, 1978). When a cutting is made, the living cells at the cut surface are exposed to the environment, and the physiological unity of the plant is disturbed, resulting in a redistribution of substances, including plant hormones (HARTMANN AND KESTER, 1983). The first response of the stem tissue to the wound stimulus is the suberization of the parenchymatous tissue located in the immediate proximity of the terminal and basal cuts (MAHLSTEDE AND HABER, 1957). This involves the oxidation of fatty substances located in, and secreted into, the superficial layers of cells adjacent to the cut surface. Suberization requires suitable conditions of

aeration. This process is aided significantly by the alkaline reaction of the cell sap, and occurs first at the basal cut. It is thus thought to be hormonally controlled (MAHLSTEDE AND HABER, 1957). The process results in the formation of a necrotic plate sealing the wound.

During the sealing of the wound by the formation of suberin in the living stem tissue, the non-living vessels in the xylem region may be blocked by a wound gum to prevent desiccation. This occurs primarily in cuttings collected in autumn (late September and October) (MAHLSTEDE AND HABER, 1957).

Poor aeration of the rooting medium, as well as immature tissues may prevent the formation of suberin, resulting in the entry of fungi or bacteria to the basal portion of the cutting and a typical damping off reaction may occur (MAHLSTEDE AND HABER, 1957).

The formation of suberin and gum form temporary protective layers, aimed at preventing the access of pathogenic organisms. These chemical barriers are replaced by an internal cork or wound cork region. The differentiated and therefore specialized cells beneath the wound surface are incapable of cell division. These cells must thus dedifferentiate, lose their original functions, regain mitotic activity, and ultimately form meristematic tissues (BARCKHAUSEN, 1978). Cork cells, originating from the cambial layer, develop parallel to the cut surface in a continuous layer immediately below the suberin deposit, and function as a cork phellogen. This then produces a periderm to minimize water loss and provide a natural barrier against micro-organisms (MAHLSTEDE AND HABER, 1957). This meristem or wound cambium, in the course of a new differentiation phase, will replace the damaged tissue (BARCKHAUSEN, 1978).

A third wound healing response, callus formation, is evident after three or four weeks. If cuttings are stored instead of benched, callus formation proceeds at a rate proportional to the temperature. Researchers generally

agree that callus formation and root production are two independent processes, although the two are usually collateral (MAHLSTEDE AND HABER, 1957). Certain cells in the vicinity of the vascular cambium and phloem begin to divide and initiate adventitious roots (HARTMANN AND KESTER, 1983). Adventitious roots may be produced from callus tissue under optimal environmental conditions, but only from older, suberized callus cells. The initiation and development of both roots and callus is influenced by the conditions both within the cutting and external to it (MAHLSTEDE AND HABER, 1957).

Under environmental conditions favourable for rooting, callus usually forms at the basal end of the cutting, as a result of the division of living cells behind the necrotic plate (HARTMANN AND KESTER, 1983). This is a proliferation of parenchymatous tissue, formed from young cells at the base of the cutting in the region of the vascular cambium, although the cells of the cortex, pith and phloem may also be involved (MAHLSTEDE AND HABER, 1957). The practice of splitting the cutting base longitudinally causes increased callus development in the presence of IBA, and enhances rooting associated with the new cambium formed in the callus (HOWARD, HARRISON-MURRAY AND FENLON, 1983).

The first roots often appear through this callus, although these two processes are usually independent of each other. In some species such as *Hedera helix* (adult), adventitious roots form in the callus. Both are, however, dependent on similar environmental conditions and therefore appear simultaneously (HARTMANN AND KESTER, 1983). Cells adjacent to those injured on the exposed surface of the cutting enlarge as a result of the increased food reserve gradient and undergo a series of meristematic divisions to produce callus. The formation of callus and the differentiation of tissues and organs, is influenced by aeration and pH, which affect the internal chemical mechanism controlling their function (MAHLSTEDE AND HABER, 1957).

1.3.3 Requirements for efficient rooting of cuttings

Well-balanced pesticide, fungicide, and fertilizer programmes, and ample soil water in the root zone, ensure maximum foliage in midsummer. This results in plants with tissues that are succulent and capable of rapid regeneration. A high carbohydrate to nitrogen ratio in the cutting favours root formation. If the nitrogen content decreases below a certain level, root formation decreases in spite of high levels of carbohydrates. Cuttings taken from vigorous shoots on young plants, high in nitrogen and low in carbohydrates thus do not root as rapidly as those taken from thin stems of older plants with high carbohydrates and medium nitrogen (MAHLSTEDE AND HABER, 1957). Shoots with a high carbohydrate to nitrogen ratio can be induced in plants by ringing and notching the stems down to the wood, thus restricting the flow of carbohydrates downwards. This also occurs in plants grown with their roots in a confined space (WRIGHT, 1973).

For most softwood cuttings the basal cut should be made half an inch below the node. This is due to the accumulation of carbohydrates at the nodes at mid-season and the anatomy of the stem segments. In addition, preformed adventitious root initials, if present, are usually distributed around the node (MAHLSTEDE AND HABER, 1957).

The extent of root production is affected by the leaf area remaining on the cutting. Water loss leading to excessive wilting can be minimised by high humidity and intermittent mist propagation systems. Cuttings with larger photosynthetic areas are less likely to die, and may produce a stronger root system in a shorter period (MAHLSTEDE AND HABER, 1957).

The presence of adventitious roots is important in the maintenance of the photosynthetic functioning of leaves (OKORO AND GRACE, 1976). The formation of adventitious roots on leaf petioles of *Populus* halted the rapid decline in protein content and extended the longevity of the leaves. The

gradual decline of the rate of photosynthesis in the hardwood cuttings of *P.tremula* was attributed to the absence of roots. Root production appeared to influence photosynthesis, not *vice versa*. Control of leaf photosynthesis was proposed by a root factor, postulated to be a cytokinin, which is translocated to the leaves where it effects the increase in the level of carboxylating enzymes (OKORO AND GRACE, 1976).

The physiological age of the plant part may also have an effect, since biochemical factors other than carbohydrate and nitrogen synthesis affect rooting (MAHLSTEDE AND HABER, 1957). Older plants have to be cut back hard first to initiate production of suitable material (WRIGHT, 1973). As a physiological condition, juvenility is characterised by both morphological features, including lack of pubescence; thinness of leaves; leaf shape modifications; thorniness, and the inability of the plant or plant part to initiate flower buds. The rooting capacity of the cutting is generally improved by selecting younger plants and initiating cuttings close to the periphery of the plant (MAHLSTEDE AND HABER, 1957).

The rooting medium of cuttings is important in that it must provide support for the cutting during the rooting process, and it must supply water and air. Most cuttings root best in a neutral medium (pH 7.0-7.2). Vermiculite fulfils these prerequisites if compaction is avoided and adequate drainage is ensured (MAHLSTEDE AND HABER, 1957). In a greenhouse, cuttings may be rooted under relatively dry conditions; after the initial watering, only syringing of the surface of the medium is required throughout the remainder of the rooting period. Temperature of the rooting medium is not of great importance (MAHLSTEDE AND HABER, 1957).

1.3.4 Necessity of adequate aeration in the rooting medium

Oxygen is necessary for the production of roots by cuttings placed in water, and observations indicate that most roots are produced at the basal end of the stem in aerated water. In non-aerated water roots are formed mostly near the surface (ZIMMERMAN, 1930). Oxygen consumption in trees (e.g. maple, ash) is most rapid in the cambial region with lower values recorded for the adjacent secondary phloem and xylem. Oxygen consumption becomes progressively lower towards the centre of the xylem, with the heartwood showing a very low basal rate. In newly formed, differentiating xylem, oxygen consumption appears to greatly exceed the cambial rate (GOODWIN AND GODDARD, 1940).

As early as 1930, (ZIMMERMAN, 1930) established that roots from willow cuttings were produced along the entire length of stems placed in a weak solution of potassium permanganate. These cuttings, as opposed to those placed in aerated water, did not show a polarity in the formation of roots. ZIMMERMAN (1930) suggested that experiments performed with both potassium permanganate and hydrogen peroxide indicate that these oxidising agents can in some way substitute for the oxygen supplied by aeration. It was not established whether free molecular oxygen is released into the water, although in the case of hydrogen peroxide this seems likely (ZIMMERMAN, 1930). Plants having preformed adventitious root primordia generally have a lower oxygen requirement for root development than cuttings that initiate and produce only adventitious roots (MAHLSTEDE AND HABER, 1957).

Both IBA and bottom heat increase respiratory activity, especially at the cutting base (HOWARD, HARRISON-MURRAY AND FENLON, 1983). Some of the effects of heterocyclic compounds, which include cytokinins and auxins, are related to hydrogen peroxide, or the promotion of the formation of free radicals (GUNSE AND ELSTNER, 1992). Cytokinins play an active role in preventing aging and senescence in plant cells. They seem to act either by

means of direct scavenging of free radicals or by preventing their formation. It can be speculated that one possible mechanism by which plant hormones act could involve the formation of free radicals. These may in turn promote the formation of either ethylene or organic peroxidases that then act as secondary messengers. It has been suggested that IAA can interact with riboflavin in the presence of light or with IAA-oxidase to form hydrogen peroxide (LESHAM, 1988, cited by GUNSE AND ELSTNER, 1992). It is possible that several N-centred radicals such as adenine-derivatives can act as regulators of ethylene formation. Ribosylation, which has been suggested as a biological mechanism regulating the activity of cytokinins, prevents the reactivity in this system. When both IAA and cytokinins are present in the same system in relative concentrations, the yield of the system seems to be controlled by IAA. This is particularly interesting with respect to adventitious root formation, which is regulated by the ratio of IAA:cytokinin (GUNSE AND ELSTNER, 1992).

1.3.5 Effect of centrifugation

Endogenous transport of rooting substances in willow cuttings can be enhanced by centrifugation (KAWASE, 1964). The basipetal centrifugation of stem cuttings enhances the formation of adventitious roots in many woody plants. KAWASE (1964) used centrifugation to concentrate rooting substances in the basal portion of willow cuttings and showed that these substances diffused into the surrounding medium during centrifugation (GOLDSCHMIDT AND MONSELISE, 1968). The diffusate collected in this manner also has a promotive effect on the rooting of both non-centrifuged cuttings and in the mung bean rooting bioassay. This diffusate is heat stable and has also been found to have a synergistic effect with IAA on the root formation of mung bean cuttings (KAWASE, 1964). It was proposed that the rooting substance(s) in the diffusate was rhizocaline. Auxins and gibberellins

have been detected in the diffusate of *Citrus* cuttings (GOLDSCHMIDT AND MONSELISE, 1968).

Dry centrifugation of *Salix babylonica* cuttings increased their rooting ability, while cuttings centrifuged in tubes containing water produced fewer roots over a longer period (VAN STADEN, 1978). Centrifugation results in the redistribution of cytokinins in the cutting with the cytokinins accumulating at the base. In the case of wet centrifugation free base cytokinins are lost to the water contained in the centrifugation tubes. The centrifugation diffusate contained compounds that co-chromatographed with zeatin-O-glucoside, zeatin riboside, zeatin and an unidentified polar compound with cytokinin-like activity (VAN STADEN, 1978).

1.4 Involvement of root stimulating substances

Apparently all substances involved in root initiation have not been identified since not all cuttings respond to the promotive effects of auxins and other known promoters or to combinations thereof (HESS, 1961b). Leaf factors, other than auxin, which induce rooting could be, at least partially, nutritional (WILSON, 1988). In *Hibiscus rosa-sinensis* L. cuttings the promotive effects of leaves were replaced by a combination of auxin, sucrose and nitrogenous compounds (VAN OVERBEEK, GORDON AND GREGORY, 1946, cited by WILSON, 1988).

Substances active in rooting include inhibitory substances. The leachate from the base of the difficult-to-root *Vitis* L.'41B' cultivar inhibited rooting when supplied to cuttings of the easy-to-root *Vitis vinifera* L. The inhibitor content in the extracts from various vines was inferred using the *Lepidium sativum* L. root growth bioassay and the *Avena* L. coleoptile growth bioassay. The difficult-to-root species and hybrids tended to have a relatively higher inhibitor content (SPEIGEL, 1954, cited by WILSON, 1988).

Potential sites for adventitious root initiation in the stem and root parenchyma are differentiated both biochemically and structurally (with respect to plastids etc.). Dedifferentiation, i.e. the loss of previously developed characteristics (ESAU, 1977), is possibly induced by a rooting morphogen, but according to WILSON, DICKS AND VAN STADEN (1994), it is more likely to be a degenerative process that increases sensitivity to a morphogen acting at a later stage (WILSON, 1994).

Root formation in cuttings was first proposed to be promoted by a substance called rhizocaline, a thermostable substance produced by the leaves in the presence of light. This was later found to be auxin but the name rhizocaline was suggested instead by Went and Bouillenne, in 1933 (HESS, 1961b), for the root-forming substances found in the cotyledons of *Impatiens balsamina* L., and by Went in 1938 (KAWASE, 1964) for another hormone-like substance that was produced in the leaves and stored in the cotyledons. The basipetal transport of this substance was as a result of the auxin gradient established by the polar movement of auxin. This substance had to be present together with IAA to allow root formation (KAWASE, 1964). Although rhizocaline has been proposed to be a non-specific carbohydrate or nitrogenous compound, and has been thought to be Vitamin K or biotin, research showed no relationship between the unknown rooting substances and amino acids from casein hydrolysate, yeast vitamins or ammonium sulphate (KAWASE, 1964).

BOUILLENNE AND BOUILLENNE-WALRAND (1955) proposed that rhizocaline consists of the product of a reaction between auxin and an orthodiphenolic, catalysed by an oxidase enzyme (cited by WILSON, 1988). Phenolics do promote rooting in some plants (WILSON, 1988). It was hypothesized that orthodiphenolics react directly with auxin to form one or more auxin-phenolic conjugates that then create the predisposition to root (HAISSIG, 1974, cited by WILSON, 1994).

Conditions inducing root initiation vary from site to site and with environment. Cells at potential sites for root initiation are not identical, differing, even within the same cell type, as a result of variation in lineage, ontogeny (including autolysis, the stage in the cell cycle, cell anatomy, ploidy) and their position relative to other cells (WILSON, 1994). Since, according to TREWAVAS (1982), cells vary in their sensitivity to plant hormones, potential sites for root initiation may also vary in their sensitivity to the rooting morphogen. The root stimulating morphogen thus may be both uniformly and abundantly distributed in the base of the cutting. This sensitivity could be the factor limiting rooting through the restriction of the number of potential sites for root initiation, not the presence or concentration of the morphogen itself (WILSON, 1994).

Physiologically active substances were released by rooting willow cuttings into the solution or sand medium in which rooting had occurred. The active substance was thermostable and soluble in water, and when applied to unrooted willow cuttings subsequently affected the number and length of roots produced. Stimulation of root growth in dormant cuttings was observed while the effects on non-dormant material were mostly injurious. Stimulation or inhibition was related to the concentration of the liberated material. The effects of the active substance were similar to those of synthetic growth stimulants (GRACE, 1945). The amount and composition of active substances varied with the time of collection of the source cutting (GRACE, 1945).

HESS (1961) isolated four root promoting substances in the easy-to-root juvenile *Hedera helix* L. and the red flowering *Hibiscus rosa-sinensis* L. (HESS, 1961a, 1961b). These compounds, as a group, were soluble in water and methyl and ethyl alcohol, and insoluble in chloroform and ether. Root promoting substances are thermostable and non-diolytic (i.e. fairly large molecular size) (HESS, 1960). Of these four compounds, one was thought to be a phenolic compound (referred to as cofactor 4) (HESS, 1961a). These four substances were found in the juvenile forms of *Hedera helix* while the

mature tissue either lacked these substances or contained smaller quantities (HESS, 1961a).

The presence of a non-auxin endogenous "root-forming stimulus" (ERS) was shown by HAISSIG AND RIEMENSCHNEIDER (1992). This endogenous factor acts additively with auxin (i.e. its effect is not replaced by auxin) in *Pinus banksiana* seedling cuttings. These researchers could not demonstrate whether this ERS is a chemical stimulus or a biophysical or anatomical one. The presence of such a factor could explain the ability of a juvenile plant cutting to root, as opposed to the limited rooting ability of the cuttings from the mature plant (HAISSIG AND RIEMENSCHNEIDER, 1992).

WILSON (1994) concludes a review of this subject by stating that even if it were possible to chemically define the rooting morphogen, this is probably irrelevant. The complexity of interactions within the cell make identifying the rate-limiting step, which need not necessarily exist, or may vary according to conditions, difficult. The activity in the cutting prior to the action of the switch from stem to root would be more important than the mechanism of the change itself (WILSON, 1994).

1.4.1 The role of plant hormones

The greatest contribution of hormones to plant propagation lies in their success in bringing about earlier rooting and sturdier root systems in the cuttings of many species of deciduous flowering shrubs and broadleafed evergreens. Hormone treatments also increase the percentage of rooting in difficult to root cuttings. The propagation of many commercially important herbaceous plants and a number of the more common species of coniferous trees and shrubs is also facilitated (AVERY AND JOHNSON, 1947).

The formation of roots by cuttings is as a result of a delicate balance between stimulative and inhibitory endogenous factors (WIESMAN AND RIOV 1994). IAA is a major component in this balance, but if any one factor is limiting (e.g. environmental, nutritional, co-factor), the whole complex becomes ineffective in triggering root formation (MULLINS, 1972). Auxins, which dominate the rooting process of cuttings, stimulate rooting, while cytokinins and gibberellins inhibit it. Exogenously applied IAA and ABA were found to cause a 2-3 fold increase in the number of roots produced by mung bean cuttings, while kinetin and gibberellins suppressed root initiation (CHANDRA, GREGORY AND WORLEY, 1971). Although IBA is widely used commercially to stimulate rooting, in many cases it is not satisfactory (WIESMAN AND RIOV 1994).

Growth retardants can stimulate rooting of cuttings, in particular, growth retardants of the triazole group (e.g. paclobutrazol) are effective in inducing adventitious root formation. The mechanism involved in this process is not clear, and it has been suggested that these substances may antagonize the inhibitory effect of gibberellins, or may promote rooting indirectly by retarding shoot growth and thereby diverting the partitioning of assimilates and/or hormones to the base of the cuttings (WIESMAN AND RIOV 1994).

1.4.2 Auxins

The main hormones directly promoting root formation are auxins (GASPAR AND HOFINGER, 1988). Auxins are the only group of chemicals that consistently enhance root formation in easy-to-root cuttings (CROUCH, 1990). IBA, dispensed in powder (talc) or liquid preparations, enhances the rooting of cuttings. Liquid IBA formulations are often more effective. High concentrations ($\geq$ 1% IBA) may induce rooting of many difficult-to-root woody species (CHONG, ALLEN AND BARNES, 1992; CHONG AND HAMERSMA, 1995).

A high level of endogenous auxin has been causally related to the initiation of adventitious root primordia (CROUCH, 1990). Auxin plays a central role in lateral root and adventitious root formation (PELOSI, LEE, CHANDLER AND HAMILL, 1995). There is, however, no direct evidence for the mode of action of auxins or co-factors in the control or direction of the rooting process. This is due to the difficulty of measuring events that are localized in a small number of cells, and of isolating key factors from a complex set of biochemical interactions (HAND, 1994). Mixtures of hormones (e.g. IBA and NAA) are more effective than equivalent concentrations of a single hormone for rooting cuttings of a number of species (AVERY AND JOHNSON, 1947).

In *Salix fragilis* L. cuttings, the application of IAA stimulated the earliest cell divisions of the root primordia without affecting root initiation. Auxin may act as a rooting promoter by locally increasing the activity of a rooting morphogen (WILSON, 1994). The severance of cuttings from the mother plant induced DNA synthesis and cell division locally throughout mung bean hypocotyl cuttings. These sites only developed further into root primordia if IAA was supplied (WILSON, 1994). It has been suggested that there are two phases of auxin action during root formation, the first of which can be induced by chemicals other than IAA. Phases of adventitious root formation that show varying sensitivities to auxin and other chemicals have been identified (JARVIS, 1986). The second phase, which culminates in the formation of root primordia, is dependent on the presence of auxin, at least in the latter stages (JARVIS, 1986).

The wound response generated in a cutting could stimulate cell autolysis (WILSON AND VAN STADEN, 1990). Autolysis represents the dedifferentiation or senescence of cells, and is necessarily followed by synthetic or anabolic processes in the formation of roots. Since IAA is a product of protein catabolism, autolytic processes could cause the *in situ* production of IAA, and reduce the level of differentiation (WILSON, 1994).

Frequently maximum rooting occurs when high levels of auxin are applied immediately or soon after cuttings are made (JARVIS, 1986). The concentrations used, are just below those that induce symptoms of toxicity (HARTMANN AND KESTER, 1983; CROUCH, 1990). Too high a concentration of auxin may have a deleterious effect on the number of roots produced, due to the death of the cortical tissue (THIMANN, 1977). Injuries caused by toxic concentrations of hormones are indicated by a yellowing and loss of leaves, checking of bud growth, poor callus formation, and a blackening and eventual killing of the base of the stem. Softwood cuttings of deciduous shrubs are more susceptible to injury by high concentrations of hormones than are cuttings of herbaceous plants. Similarly, the very woody species are more readily injured than the less woody (AVERY AND JOHNSON, 1947).

Other observations that indirectly implicate auxin in the control of adventitious root formation are:

1. Young leaves and active buds (sources of auxin) enhance rooting in some cuttings. Exogenously applied auxin completely, or at least partially, replaces these promotive effects.
2. Auxin is transported preferentially in a basipetal direction in the root.
3. Compounds interfering with auxin transport or action (e.g. triiodobenzoic acid (TIBA)) inhibit root regeneration while chemicals that affect auxin metabolism (eg. phenolics), often improve the rooting response of the cutting (CROUCH, 1990).

1.4.3 Cytokinins

Adventitious root formation is induced in some plants by the removal of the roots. As opposed to auxins, cytokinins are considered to be inhibitors of adventitious root formation (BOLLMARK AND ELIASSON, 1986).

Cytokinins are characterized by their cell division-inducing activity in the tobacco pith bioassay (in the presence of auxin). In addition, they must be N^6-substituted adenines, i.e. derivatives of the nucleic purine base (LESHEM, 1973). Substances differing from this structure but exhibiting the required activity in the bioassay are described as "cytokinin-like" (YOPP, AUNG AND STEFFENS, 1986). Other substances promoting cell division include diphenyl urea and derivatives of nicotinamide (LESHAM, 1973).

Cytokinins are ubiquitous in both higher and lower plants, are produced by microorganisms, and are secreted by certain insects (LETHAM, 1978). They occur in free or bound forms in tissues and cells. Bound cytokinins are associated with tRNA (VAN STADEN AND HARTY, 1988). Cytokinin bases in tRNA are of functional significance and the modification of these bases can affect the ability of tRNA to function in protein synthesis. It is not known if the growth promoting ability of cytokinins is related to their presence in tRNA (LETHAM, 1978). Cytokinin biosynthesis occurs in tissues that are meristematic, or at least still retain growth potential. Cytokinins are synthesized predominantly in the roots and are transported acropetally in shoots (MOORE, 1979) in the form of cytokinin ribosides (CROUCH, 1993) via the transpiration stream.

The production of free cytokinins during the growth and development of the plant can be accounted for either by the turnover of cytokinin-containing tRNA, by *de novo* synthesis, or by a combination of the two mechanisms (KOSHIMIZU AND IWAMURA, 1986). The characteristics of the N^6-side chain can greatly influence biological activity. These include chain length, the degree of saturation and configuration (SKOOG AND LEONARD, 1968).

The mode of action of cytokinins, the level or site at which they are active in the cell, has not been fully elucidated yet. It is not known whether *in vivo* cytokinin activity occurs specifically at the level of the cytokinin base, riboside or ribotide. Cytokinins may only become active after metabolic

transformation into other substances (CROUCH, 1993). Cytokinins appear to affect plant metabolism as mediators, promoters or inhibitors of growth at a level close to, although not necessarily at, the genome (VAN STADEN AND DAVEY, 1979). Some effects of cytokinins can be related directly to the ability of cytokinins to regulate expression of specific genes (KAMÍNEK, 1992).

Zeatin is thought to be the most active of the known cytokinins, and together with its metabolites seem to make up the bulk of free cytokinins found in the vegetative and reproductive parts of plants. It was suggested that iso-pentenyladenine acts as a precursor for zeatin (LETHAM AND PALNI, 1983). Benzylaminopurine (BA) has recently been found to occur naturally in limited abundance and distribution (NANDI, LETHAM, PALNI, WONG AND SUMMONS, 1989). Cytokinin O-glucosides, which include zeatin-O-glucoside [Z-OG], zeatin-O-glucoside-9-riboside [ZR-OG], dihydrozeatin-O-glucoside [DHZ-OG] and dihydrozeatin-O-glucoside-9-riboside [DHZR-OG], have been conclusively identified in a number of plant tissues (LETHAM AND PALNI, 1983).

Ribotide formation appears to be associated with cytokinin uptake and transport across cell membranes. When cytokinin bases are exogenously applied to tobacco cells, the primary metabolites are initially cytokinin ribotides, which accumulate in cells that are impermeable to them (KOSHIMIZU AND IWAMURA, 1986). LETHAM AND PALNI (1983) proposed the utilization of cytokinin ribosides in translocation in the xylem, O-glucosides as storage forms, nucleotides associated with the uptake of cytokinins by plant tissues and free bases as the active form. The formation of 7- and 9-glucosides, of alanine conjugates, and sidechain cleavage to adenine and its derivatives would constitute alternative mechanisms for the lowering of cytokinin activity in a tissue (LETHAM AND PALNI, 1983). This enzymic regulation of the interconversion of cytokinin ribotides, ribosides and bases thus plays a significant role in maintaining adequate levels of active

cytokinins in plant cells (KOSHIMIZU AND IWAMURA, 1986).

Metabolism of cytokinins

The metabolism of synthetic (e.g. kinetin) and naturally occurring cytokinins (e.g. zeatin, isopentenyladenine [iP]) has been widely researched (LETHAM AND PALNI, 1983). Zeatin is recognised as one of the most biologically active of the naturally occurring cytokinins, and is rapidly metabolised by plant tissues (BLAKESLEY AND LENTON, 1987). Cytokinin bases, nucleosides and nucleotides appear to be readily interconvertible within plant tissues. The enzymes responsible for catalysing these interconversions are not likely to be cytokinin-specific, although in some tissues specific enzymes may be present. The extent to which one-step or two-step phosphoribosylation of cytokinin bases occurs appears to depend on the plant system under investigation (CROUCH, 1993).

Cytokinin oxidase is the enzyme that catalyses the cleavage of the N^6-isoprenoid side chain. Much of the exogenously applied zeatin, isopentenyladenine, zeatin-9-riboside (ZR), and isopentenyladenosine (iPA) - all compounds with a double bond in the N^6 side chain - undergo oxidative side chain cleavage to give adenine, adenosine and adenine nucleotides (McGAW, SCOTT AND HORGAN, 1984; McGAW, 1987). This leads to the irreversible loss of cytokinin activity, a factor that is important in the regulation of cytokinin activity levels (McGAW, 1987). In contrast, BA is not affected by this enzyme (BLAKESLEY AND LENTON, 1987).

The catabolism of cytokinin in plant tissues seems to be predominantly due to the activity of cytokinin oxidase. This enzyme catalyses the oxidation of cytokinin substrates with unsaturated isoprenoid side chains. This process utilises molecular oxygen as the oxidant (HARE AND VAN STADEN, 1994). Adenine and its derivatives (adenosine and adenine nucleotides) are the major metabolites following the metabolism of Z, ZR, iP and iPA, facilitated by

cytokinin oxidase (LETHAM AND PALNI, 1983). This leads to an irreversible loss of cytokinin activity (McGAW, 1987). Stability, and resistance to enzymic attack are enhanced by isoprenoid sidechain reduction, glucosylation and alanine conjugation, as well as binding to proteins (LETHAM AND PALNI, 1983).

Cytokinin metabolites contribute to either an active or inactive pool. Inactivation of cytokinins occurs through sidechain cleavage or alternatively through N-conjugation that proceeds through 9-alanylation or 7- and/or 9-glucosylation. 3-Glucoside is more biologically active than the other cytokinin N-glucosides and appears reversibly sequestrable, suggesting a storage role. Cytokinin glucosides are thought to be storage and/or transport forms of cytokinins depending on the physiological circumstances in various plant tissues. It is possible that cytokinin glucosides, after metabolic conversion, are responsible for active cell division and the mobilization of nutrients in dividing callus tissue. The high levels of these compounds in the phloem sap of *Salix* may result in the activity of the cambial cells (VAN STADEN AND DAVEY, 1977).

Internal levels of free, non-metabolised bases appear to be important in the initiation of physiological responses. Nucleosides and nucleotides are also considered active forms, given their ready conversion to cytokinin bases.

From a physiological viewpoint, cytokinin metabolism may be classified under three headings (HORGAN, 1987):

1. irreversible loss of biological activity through oxidative degradation of N^{6}-sidechain (products referred to as 'oxidation products');
2. irreversible conjugation with alanine or glucose with loss of or reduction in activity (products are N-conjugates);
3. reversible conjugation to (interconvertible) compounds which are themselves active, or serve as storage forms that may be converted to active cytokinins (referred to as 'active pool').

Specifically, the naturally occurring iP- and/or Z-type cytokinins can be converted to a variety of metabolites via one or more of the following basic reactions:

1. *trans*-hydroxylation of the terminal methyl group on the side chain of iP-type cytokinins;
2. side chain reduction of Z-type cytokinins;
3. isoprenoid side chain cleavage that leads to the irreversible destruction of activity by cytokinin oxidase;
4. *O*-glucosylation (O-glucosides and their ribosides are easily hydrolysed by β-glucosidase with the release of free cytokinins);
5. *N*-glucosylation;
6. ring substitution by alanine moiety;
7. base-ribonucleoside-ribonucleotide interconversions (KOSHIMIZU AND IWAMURA, 1986).

Exogenously applied cytokinin supplied to callus rapidly degrades, or at least undergoes some molecular alteration. Dihydrozeatin resists side chain cleavage but is able to convert to dihydrozeatinriboside (DHZR) and to the O-glucosides of dihydrozeatin and dihydrozeatinriboside. Zeatin metabolism is associated with side-chain cleavage rather than riboside or glucoside formation (FORSYTH AND VAN STADEN, 1986). Cytokinin bases and ribosides are subject to rapid and extensive metabolism in some plant tissues, including those used in bioassays. While LETHAM AND PALNI (1983) report no studies on the metabolism of exogenous cytokinin nucleotides, the nucleotides formed from supplied cytokinin bases and ribosides appear to be metabolised rapidly. The 7-glucosides of cytokinins, and some 9-glucosides, show great metabolic stability, in among others soybean callus tissue (LETHAM AND PALNI, 1983).

Thus the metabolism of exogenously applied naturally occurring, and synthetic cytokinins, must be considered as an important component in the assessment of the biological activity of these compounds. The role of

metabolic deactivation in moderating biological activity is clearly important, more so than metabolic activation. Limited knowledge of the metabolism of cytokinins prevents the construction of a clear model for the relationship of metabolism with the expression of biological activity (McGAW, SCOTT AND HORGAN, 1984).

Roles of cytokinins in plant development

Cytokinins play a role in other stages of plant development, e.g. cell enlargement and cell differentiation, and the in the flow of nutrients and assimilates through the plant (VAN STADEN AND HARTY, 1988). Cytokinins are able to retard senescence in both the intact plant and excised plant parts (possibly via protein synthesis), and to influence nutrient mobilization within plant tissues (VAN STADEN AND DAVEY, 1979). Application of cytokinins promotes the growth of lateral buds by reducing the dominance of the apical bud. In addition, cytokinins enhance resistance to various stresses (including salinity, moisture, high temperature), and regulate plant growth under drought conditions (KAMÍNEK, 1992). With respect to plant metabolism, they have been shown to affect enzyme activity, the biosynthesis of growth factors, and the appearance and disappearance of cell organelles (VAN STADEN AND HARTY, 1988).

Virtually all plant development processes seem to be governed by the concerted and mutually modifying regulatory action of several hormones (HEIDE, 1972). It has been proposed that cytokinins regulate growth and development by controlling the rate of protein synthesis (ELLIOT, HOSFORD, LENTON, MILFORD, POCOCK, SMITH, LAWRENCE, AND FIRBY, 1983). In senescing tissues, levels of DNA, RNA and protein gradually decline, so that the RNA:DNA ratio changes. Cytokinins retard these processes, and can stabilize or even promote RNA:DNA ratios, thus indicating that RNA metabolism may be directly involved (LESHAM, 1973). Cellular cytokinin levels peak shortly before cytokinesis, concurrently with increased protein

and DNA levels. This indicates the importance of cytokinins in the process of cambial initiation (ELLIOT, HOSFORD, LENTON, MILFORD, POCOCK, SMITH, LAWRENCE, AND FIRBY, 1983).

The characteristic stimulative function of cytokinin in cell division and callus growth in plant tissue cultures, is closely related to the function of cytokinins in organ formation in plant tissues. These processes, like most other hormonally controlled processes, are dependent on the delicate and quantitative interaction between cytokinins and other phytohormones, especially auxins (HEIDE, 1972).

Action of growth retardants

Growth retardants have been shown to stimulate root growth. Plant growth regulators such as daminozide, paclobutrazol and triadimelon act synergistically with IBA on the formation of adventitious roots in mung bean hypocotyl cuttings. Individually these growth retardants stimulate root growth, but to a lesser extent than IBA. Treatments of IBA, followed 24 hours later by the growth retardant, and treatments of the growth retardant in combination with IBA, were more effective than IBA alone (PAN AND ZHAO, 1994).

These growth retardants inhibit the biosynthesis of gibberellins (GA), thus deceasing endogenous levels of GA. Application of GA inhibits adventitious root formation in cuttings of a variety of species, including mung beans. The induction phase of root primordia is promoted by IAA accumulation, while the early initiation phase is inhibited by GA. Rooting induced by IBA or growth retardants could be reduced by GA. Application of IBA and the growth retardant thus could have a synergistic effect on rooting when applied simultaneously or sequentially (PAN AND ZHAO, 1994).

Paclobutrazol and thidiazuron are well-known bioregulators. Paclobutrazol, a member of the triazole family, interacts with IBA to promote rooting in cuttings (WEISMAN AND RIOV, 1994). Paclobutrazol is most promotive to root formation when applied to the cutting immediately after preparation, with IBA. The effect seems to lie in the cell division phase of the adventitious root meristem (WEISMAN AND RIOV, 1994).

Paclobutrazol acts to reduce the endogenous levels of gibberellins, a phytohormone known to inhibit cell division. WIESMAN AND RIOV (1994) proposed that the contribution of paclobutrazol is to stimulate cell division, while IBA influences primordia organisation, resulting in a dramatic stimulation of adventitious root formation by this combined treatment. Another alternative is that paclobutrazol has a direct influence on IBA metabolism, which is known to have an effect on the rooting of cuttings. Paclobutrazol is also thought to increase the sink capacity for assimilates in the base of the cuttings, thereby enhancing the root induction potential of IBA (WIESMAN AND RIOV, 1994).

Further research has shown that paclobutrazol appears to mimic the effect of auxin by irreversibly inducing root initiation on the first day of culture. The effect of the application of paclobutrazol is similar to that of exogenous IAA in that roots are initiated only at the cut surface. It is suggested that both stimulants require the accumulation of endogenous IAA at the basal cut for root induction (LESHAM, RONEN AND LURIE, 1994).

1.4.4 Ethylene

Ethylene is an endogenous plant hormone involved in the growth, differentiation and aging of most, if not all, plants (LIEBERMAN AND KUNISHI, 1972; WANG, HO AND SU, 1994). Ethylene has been implicated in the rooting response (McCOWN, 1988). In the literature, the effect of

ethylene on the formation of adventitious roots in various plant cuttings and seedlings yielded differing results - some stimulative, some inhibitory and some showing no effect (WANG, HO AND SU, 1994).

Almost all plant tissues are capable of high levels of ethylene production in response to wounding, stress or the appropriate hormonal stimulus (OSBORNE, 1978), including auxins (YANG AND PRATT, 1978). Rates of ethylene production in growing or expanding tissues have been linked to levels of endogenous auxin. Application of auxin to isolated plant parts invariably enhances the evolution of ethylene (OSBORNE, 1978).

Ethylene production in response to stress

While many plant tissues appear capable of synthesizing ethylene, synthesis is suppressed by some unknown control mechanism in unstressed tissues. Wounding or other stresses relieve this constraint resulting in an increase in ethylene production, which in turn may activate a variety of metabolic systems (YANG AND PRATT, 1978). This may include the activation of a system enabling the plant to cope with the initial stress or to eliminate it. Accelerated senescence and abscission are two of the known physiological consequences of stress. Stress-ethylene may be involved in wound-healing; the production of phytoalexins and increases in disease resistance, however these responses are not general phenomena. Some of the physiological differences known to exist between excised tissue and intact organs are attributable to the stress-ethylene induced by cutting. Stress-ethylene may also influence respiratory and metabolic activity and act directly in wound healing. Wounding is often accompanied by an increase in respiration and in ethylene production (YANG AND PRATT, 1978).

Stress-ethylene is of metabolic origin, and its production may be great or small depending on the tissue, or the nature and intensity of the stress. The biosynthetic pathway of stress-ethylene does not appear to differ from the

normal ethylene pathway and methionine is a common precursor (YANG AND PRATT, 1978).

The production of stress ethylene by damaged tissues has two patterns:

1. In tissue excised from underground storage organs, ethylene production quickly increases to a maximum soon after cutting, and then declines to a steady, slow rate. In such tissues, stress ethylene therefore activates only those metabolic systems that are capable of responding to low concentrations of the gas.
2. In other tissues, an induced rise in ethylene production develops after a lag period, and the concentration of stress-ethylene in these tissues is so great it can activate a number of metabolic systems (YANG AND PRATT, 1978).

Due to the presence of the double bond, ethylene reacts readily with halogens to form substituted hydrocarbons and other saturated addition products. Ethylene readily undergoes oxidation. The reaction with potassium permanganate results in the oxidation of ethylene to ethylene glycol and the formation of manganese oxide (MnO_2) which is toxic to plant tissues and under certain circumstances reduces the effects of applied ethylene. No evidence shows that ethylene oxide is a specific inhibitor of ethylene in plants (OSBORNE, 1978).

Effect of ethylene on adventitious root formation

Treatments stimulating root formation (e.g. auxin application or wounding) are known to stimulate ethylene production. Some phenolic compounds that stimulate rooting have been implicated in the regulation of ethylene biosynthesis. With respect to lateral root production, ethylene inhibits the formation of adventitious root primordia (MULLINS, 1972).

Studies on the germination of *Pisum sativum* seeds showed that the application of aminoethoxyvinylglycine (AVG) which almost completely inhibits ethylene synthesis, had a marked effect on subsequent root growth. Application of 100 μM AVG promoted radicle elongation (PETRUZZELLI, HARREN, PERRONE AND REUSS, 1995).

Both auxin and cytokinin interfere in some way with ethylene (STENLID, 1982). Ethylene production is stimulated by both auxins and cytokinins, often synergistically. It was thus thought that the inhibition of root growth caused by auxins and cytokinins was due, at least in part, to ethylene, and that stimulation of root growth was connected to inhibition of ethylene production (STENLID, 1982). Some increase in ethylene production occurs when cytokinins alone are added to plant tissues. When cytokinins and auxins are supplied together to mung bean hypocotyls, production of ethylene is greatly increased, and can be related directly to a cytokinin suppression of the conjugation of auxin to give IAA-aspartate and the maintenance of high levels of free IAA. There is a clear enhancement of auxin-induced ethylene production in the presence of kinetin in intact *Xanthium* leaves. A reduction in the rate of the metabolism of endogenous IAA, and an increase in ethylene production would result from an enhanced level of endogenous cytokinin. Other factors may also be involved (OSBORNE, 1978).

Substances of varied chemical structure were found to promote the elongation of roots and counteract the effect of auxins and cytokinins. This was used to support the view that the inhibitory action of auxins and cytokinins is related to the synthesis and action of ethylene (STENLID, 1982), which acts antagonistically to the growth hormones IAA, cytokinins and gibberellins (LIEBERMAN AND KUNISHI, 1972). The function of ethylene may be to moderate the action of growth hormones, keeping production to within normal bounds and thus prevent excessive growth. Ethylene may act by suppressing the synthesis of IAA and cytokinins, or by suppressing the effect of IAA, gibberellins or cytokinins at their site of action (LIEBERMAN AND

KUNISHI, 1972). Ethylene synthesis is affected by free radicals. Substances such as benzoic acid, salicylic acid and n-propylgalate (acting as free radical scavengers) reduce ethylene production and have been shown to stimulate root elongation, and reduce the inhibition caused by auxins and cytokinins (STENLID, 1982).

Research conducted by WANG, HO AND SU (1994) showed that adventitious root formation was greatly influenced by ethylene. Ethylene depletion increased root density and ratio. An initial high rate of ethylene production induces the formation of adventitious roots. When the ethylene concentration increased in the medium surrounding the root-producing calli, retardation of root formation was observed. Ethylene is thus only a trigger and its extended presence in culture, even at low concentrations, exerts an inhibitory influence (WANG, HO AND SU, 1994).

The inhibition of root elongation *in vivo* and in isolated root tips by the application of auxin (in moderate concentrations and low volumes), was thought to be mediated by IAA-stimulated ethylene production (CHADWICK AND BURG, 1970). This could constitute a feedback system in which the action of auxin is modified by a hormone induced regulator (CHADWICK AND BURG, 1967). ANDREAE, VENIS, JURSK AND DUMAS (1968), however, argue that while ethylene is certainly produced in response to IAA, the effects of applied ethylene and of IAA are distinct in so many ways that it seems doubtful whether the very small amounts of ethylene produced can be of major significance in mediating the inhibitory effect of IAA.

Stimulation of growth in lentil root tips by low concentrations of auxin was accompanied by reduced ethylene production, but growth inhibition by high levels of auxin induced a diminished ethylene evolution. DUBUCQ, HOFINGER AND GASPAR (1978), assert the non-dependency of auxin-induced root growth inhibition on ethylene production, and propose that the ethylene evolved upon treatment with inhibitory concentrations of IAA, is the result of

inhibition and not the cause. The diversity of stimuli-inducing ethylene production suggests that its production is a non-specific response to the disruption of normal metabolism and that treatment with auxin may represent such a disruption (DUBUCQ, HOFINGER AND GASPAR, 1978).

1.4.5 The significance of hormone ratios and interactions

The auxin/cytokinin interaction hypothesis proposed by SKOOG AND MILLER in 1957 (cited by HEIDE, 1972), has been shown to have general validity in *in vitro* plant tissue and organ cultures (HEIDE, 1972). A high auxin to cytokinin concentration ratio in the culture medium induces root formation, while the reverse ratio favours bud and shoot formation (KAMÍNEK, 1992). There is, however, limited information available regarding the *in vivo* operation of such an interaction system. *In vivo* interactions of auxin and cytokinin were investigated in *Begonia* leaf cuttings. Isolated leaves of *Begonia x cheimantha* cultivated at different temperatures resulted in differing regenerative ability. Root formation was rapid and abundant at relatively high temperatures. Similarly with respect to photoperiod, it was found that short days reduce root formation. These effects are thought to be mediated by changes in the endogenous levels of cytokinins and auxins. An increase in the auxin to cytokinin ratio at high temperatures and long days, causing changes in the level of one or both compounds, could be consistent with the Skoog and Miller hypothesis and would be able to account for the observed environmental effects (HEIDE, 1972).

Cytokinin, when supplied together with auxin at moderately high concentrations (10^{-5} M), inhibits root formation. At low concentrations (10^{-8} M) this has a slight stimulative effect (WIGHTMAN, SCHNEIDER AND THIMANN, 1980). This seems to imply that a certain minimum level of cytokinin is essential for the induction of adventitious rooting. In the intact plant the continuous transport in the transpiration stream of cytokinins

synthesized in the root would prevent adventitious root formation. The cessation of this supply due to the removal of the roots would result in a lower concentration of cytokinins, and subsequent root initiation (BOLLMARK AND ELIASSEN, 1986).

The results described by HEIDE (1972) showed a pronounced interaction of IAA and kinetin, and that an increase in the kinetin to IAA ratio had the same effect on bud and root formation as a lowering of the temperature or shortening of the day-length. It thus appears that temperature and day-length effects can be imitated by varying the auxin to cytokinin ratios (HEIDE, 1972). Cytokinins at high concentrations stimulated bud formation and inhibited root formation. Auxins, at similar concentrations (160 μM), had the opposite effect (HEIDE, 1965). There is thus strong evidence for the existence of the *in vivo* interaction of auxins and cytokinins in the regeneration of adventitious buds and roots in *Begonia* leaves (HEIDE, 1972). Cytokinins hinder conjugation of IAA (GOODWIN, 1978; STENLID, 1982).

Auxin and cytokinin are necessary for the competence and determination of tissue explants to form roots. Root inducing factors are auxins, or auxins in combination with relatively low levels of cytokinins (MOHNEN, 1994).

It was suggested that gene activation, presumed to take place after the isolation of the cutting (leaves or other organs), is differentially determined by the environment to which they are exposed subsequent to the isolation (HEIDE, 1972). Stem cells must alter their functioning to give rise to adventitious roots. This would suggest that an autonomous entity such as a gene, nucleus, whole cell or group of cells is influenced by a rooting factor, or an external factor to effect this change (WILSON, 1994).

Regeneration is subject to modification by other growth substances, e.g. gibberellins. Both bud and root formation in *Begonia* leaves were strongly inhibited by gibberellins. This inhibitory action by gibberellins could not be

reversed by cytokinins and auxins respectively (HEIDE, 1972).

1.5 The effects of cytokinins on the rooting of cuttings

Cytokinins are considered to be natural inhibitors and regulators of root growth (STENLID, 1982). The application of cytokinins to stem cuttings generally inhibits adventitious root formation, the early stages being the most sensitive to inhibition (BATTEN AND GOODWIN, 1978). Examples where the application of cytokinins has promoted adventitious root formation are rare. The reason for the apparent discrepancy between cytokinins is not clear, and needs further investigation with respect to the rooting process that can be divided into:

1. an induction and root meristem formation phase; and
2. an elongation phase (VAN STADEN AND HARTY, 1988).

Cuttings with a naturally high cytokinin content are more difficult to root than those with a low content (OKORO AND GRACE, 1978). In general, applied cytokinins inhibit root initiation in stem cuttings. Cytokinins, at very low concentrations, have however, been found to promote root initiation when applied at an early developmental stage to decapitated pea cuttings or together with auxins to *Begonia* leaf cuttings. When applied at higher concentrations cytokinins inhibited root formation (ERIKSEN, 1974).

Exogenously applied cytokinins inhibit root formation in cuttings and antagonize the stimulative effect on rooting of abscisic acid in *Populus alba* (OKORO AND GRACE, 1978). HEIDE (1965), however, found that at very low concentrations cytokinins could stimulate the effect of auxin on rooting. ERIKSEN (1974) suggested a synergistic interaction between auxin and cytokinin.

Cytokinins inhibit root growth when supplied to the roots of onion, barley, *Brassica*, *Isatis*, and tomato. In *Pisum*, *Triticum and Zea*, zeatin (like auxin) inhibits root growth by reducing the duration of the elongation phase. In onion, kinetin inhibits mitosis in the apical meristem (GOODWIN, 1978).

Most cytokinins give an optimal response with respect to cell division in the range of 10^{-5} to 10^{-6} M. Investigations into the initiation and emergence of lateral roots showed that all the cytokinins tested, strongly inhibited this process at concentrations above 10^{-6} M, except iP and kinetin which exhibited inhibition at 10^{-5} M and above. Synthetic cytokinins are less inhibitory than the natural cytokinins, being promotive at 10-fold higher concentrations. This effect may be due to a faster rate of metabolism (VAN STADEN AND HARTY, 1988).

Applied cytokinins specifically inhibit root elongation (BOURQUIN AND PILET, 1990). Root elongation in wheat (*Triticum aesitivum*), flax (*Linum usitatissimum*) and cucumber (*Cucumis sativus*) seedlings is inhibited by both endogenous and synthetic cytokinins including kinetin, benzyladenine, iP, zeatin and their 9-ribosides. Concentrations as low as $\pm 10^{-9}$ M of zeatin and iP produced a 50% inhibition of rooting in wheat and flax respectively. The ribosides of these cytokinins were less inhibitory (STENLID, 1982).

Zeatin, the most active cytokinin in cell division bioassays, was the most inhibitory of the natural cytokinins tested. The work conducted by STENLID (1982) on the effect of several cytokinins on wheat roots showed that zeatin exhibits the most significant inhibition of growth. This was reported after a treatment period of 18 - 20 hours. Later work (BOURQUIN AND PILET, 1990), showed a weak reactivity of roots to applied zeatin after a one-hour treatment. This led the authors to propose that the metabolism and amount of absorbed zeatin must be considered as essential experimental parameters (BOURQUIN AND PILET, 1990).

In general, all the cytokinin ribosides tested were less inhibitory than their respective free bases, with respect to root initiation and emergence as well as root elongation. These and other results seem to indicate that cytokinin ribosides may be of primary importance in the rooting process (VAN STADEN AND HARTY, 1988). Lack of supportive data in the literature could be due to the report of a blanket response for cytokinins when only one has been tested, or to the narrow range of concentrations tested (the lower concentration range has rarely been tested). Cytokinins have optimal levels that vary with organ and tissue type. Another important factor is the site of application (VAN STADEN AND HARTY, 1988).

6-Benzyladenine (BA), which has been reported to stimulate rooting, is rapidly metabolised to ribosylbenzyladenine when applied to tissue. Cytokinins such as BA have also been found to reduce the number of roots formed on primary leaf explants and their average length. This effect is alleviated to some extent by application of the cytokinin in combination with IBA. In addition, it was found that the riboside of the cytokinin applied (BA) is more inhibitory than the free base (DREWES AND VAN STADEN, 1989).

Cytokinin bases supplied to plant tissues are usually converted initially to nucleotides (BLAKESLEY AND LENTON, 1987). Investigations into the uptake and metabolism of BA have shown that almost all the BA present in the medium was taken up and rapidly metabolised to [9R]BA. Ribosylzeatin is the most abundant cytokinin exported from the roots (BLAKESLEY AND LENTON, 1987) and is present in xylem sap and stem material in relatively high levels. Centrifugation of *Salix* cuttings prior to planting resulted in the accumulation of cytokinins, largely compounds that co-eluted with ribosylzeatin, in the basal portions of cuttings which subsequently rooted better (VAN STADEN AND HARTY, 1988).

When cytokinins were applied to pea cuttings at a later stage in root initiation there was no inhibition. Experiments conducted using leaf cuttings of

Phaseolus vulgaris indicated that the synthesis and transport of cytokinins began before the new adventitious roots ruptured the epidermis of the petiole. The synthesis increased as root development progressed (FEATONBY-SMITH AND VAN STADEN, 1981).

The effect of cytokinins on root initiation thus may depend on the particular stage of initiation and on the concentration (HARTMANN AND KESTER, 1983). A low level of cytokinin is probably required for adventitious root formation. It was proposed by BATTEN AND GOODWIN (1978) that cuttings that do not respond to auxin lack sufficient endogenous cytokinins for adventitious root formation.

1.5.1 Effects of cytokinins on the stage of root development

Results obtained by ERIKSEN (1974) seem to indicate that the influence of cytokinin changes with the stage of development. There is possibly an interaction between cytokinins and other growth hormones, specifically auxin. IAA has been shown to be active in early initiation stages. Cytokinin in high concentrations may have an inhibitory effect on an early stage in rooting by blocking the activity of auxin. This is not counteracted even by high concentrations of auxin. The loss of the inhibitory effect of cytokinin during the latter phase of initiation suggests that at this stage, developing root primordia are capable of controlling the level of active cytokinin and thus do not react to the exogenous application of cytokinin (ERIKSEN, 1974). It was suggested that cytokinins are essential growth substances in this later part of the initiation phase, and that partially initiated root primordia can synthesize cytokinins, being self sufficient for this hormone (ERIKSEN, 1974). Auxin was found to have no effect at this stage (i.e. when root primordia develop into roots).

The experiments conducted by ERIKSEN (1974) in order to determine whether the cytokinin works alone, or with other growth substances, involved the application of cytokinin to either decapitated or both decapitated and debudded cuttings. It was assumed that the existence of an interaction would be revealed when the lateral bud became active. It was thus assumed that the amount of growth substance synthesised in the bud is sufficient to show this interaction.

The response to the application of BA was found to differ during the initiation phase. When BA was applied to the cuttings with no shoot meristems, there was no promotive effect. At certain concentrations BA inhibited root formation during the early phase of initiation. The promotive effects obtained following the application of BA to decapitated cuttings were of a more complicated nature. Cytokinins alone could not, however, replace the effect of the apical meristem. This effect was only apparent with decapitated cuttings, not those that were decapitated and debudded (ERIKSEN, 1974). The loss of the inhibitory effect of BA during the latter part of the initiation phase was as a result of the physiological alteration of the influence of BA on the root formation process, and was not as result of reduced transport of cytokinins (ERIKSEN, 1974).

1.5.2 Changes in the endogenous levels of cytokinins in relation to rooting

Morphological and environmental factors can affect the ease of rooting of a cutting, but this may or may not be effected through a change in the cytokinin levels (VAN STADEN AND HARTY, 1988). Cytokinin levels have been found to differ markedly between juvenile and adult plants. The levels of polar cytokinins in juvenile plants were found to be higher than in adult plants at the stage of bud activation, just prior to the breaking of dormancy. Another factor that may affect the rooting capacity of the cutting at these

stages is the qualitative and quantitative differences in the cytokinins present (HARTMANN AND KESTER, 1983).

The metabolism of cytokinins in relation to root formation and growth has been studied predominantly using leaf cuttings of *Phaseolus vulgaris. I*t is proposed that this system undergoes a continuous increase in the root to shoot ratio resulting in the accumulation of root synthates in the lamina. This allows the control of root initiation and the elimination of the effects of buds, in a photosynthetically independent organ (VAN STADEN AND HARTY, 1988).

The cytokinin content of the primary leaves of *P.vulgaris* has been analysed in relation to rooting ability. The higher levels of cytokinins, especially cytokinin glucosides, found in the leaves of 30-day old plants in comparison to 10-day old plants, were correlated with a better rooting capacity. It was concluded that the higher levels of cytokinin glucosides were used in the processes of adventitious root formation, or for the retardation of leaf senescence (VAN STADEN AND HARTY, 1988). Cytokinin glucosides may be storage and inactivation forms, and these conjugates show less activity than the corresponding bases in bioassays.

It was proposed that such storage forms are important as slow-release sources in the metabolic processes requiring the release of small, controlled amounts of cytokinins. With respect to rooting, a supposedly cytokinin-sensitive process, the conversion of free bases to these storage forms could lower the potential cytokinin activity of the tissue until the completion of those processes susceptible to high levels of free cytokinins. Reconversion to free bases could then occur, allowing their participation in the later processes where cytokinins are required (VAN STADEN AND HARTY, 1988).

Ringing *Salix babylonica* plants resulted in a decrease in the level of

cytokinins in the leaves and an increase in the bark, both above and below the girdle. Callus forms above the girdle, and in this region there was a decrease in the cytokinins tentatively identified by co-chromatography as cytokinin glucosides. This area also showed an increase in zeatin and zeatin riboside. It was proposed that these cytokinin glucosides were transported downwards and utilised and/or converted to other products at the girdle where the callus forms (VAN STADEN AND BROWN, 1977).

1.6 Aims and objectives of this study

Further research is thus needed, firstly into the determination of endogenous hormone levels, and secondly, the changes occurring during various stages of root formation. Much of the research into the role played by cytokinins in ... is contradictory. Cytokinin ribosides have, however, been ... the rooting process (VAN STADEN ... ing to VAN STADEN AND HARTY ... tative determination of endogenous ..., and the importance of the site of ... VIS, 1994).

... gate the role played by cytokinins in ... procedures can be divided into two ... and applied investigations.

... d distribution of cytokinins present in ... as conducted. A range of plants was ... tokinin levels in easy- and difficult-to-

... t various physiological stages of rooting ... primarily a qualitative determination of

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Initials

the cytokinins present using co-chromatography for tentative identification, accompanied by quantitative comparisons.

The applied investigations included a study of the effect of treatments perceived to improve the rooting capacity of cuttings. Specifically, the effects of centrifugation, and the oxidising agent potassium permanganate on the cytokinins present in the stem tissues were determined. Lastly, the effect of exogenously applied hormones - both auxins and cytokinins - was investigated. This was studied *in vivo* using the entire cutting and *in vitro* using a root culture system.

Specifically, it was determined whether the cytokinins or their metabolites are inhibitors of adventitious root formation. If this is the case, it is possible that the treatments promoting rooting, such as the oxidising agent, lowers the levels of inhibitory cytokinins (such as zeatin) in the basal area of the cutting, thus altering the ratio of cytokinin to auxin and promoting root formation.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Plant Material

The choice of stock plant was most important considering that the cuttings for the initial investigations must root readily, within a fairly short period. A range of plants was thus maintained, including both easy- and difficult-to-root species.

The genus *Impatiens*, belonging to the family Balsaminaceae, is widely distributed in tropical and subtropical regions. These herbaceous plants spread rapidly, and the perennial species can be easily propagated by cuttings or seed (PIENAAR, 1984). *Impatiens wallerana* Hook.f. is an easy-to-root and rapidly growing perennial species and was chosen for the initial experiments. A stock of these plants was available from the University Botany Department Gardens, and these, together with a stock of plants maintained in a shadehouse, were used in all experiments. A summary of the experiments conducted on *Impatiens* is presented in Figure 2.1.

Several *Eucalyptus* species, including *E. grandis* W.Hill ex Maiden, *E.macarthuri* Deane & Maiden, *E. nitens* Maiden, were grown from seedlings. Stock plants were maintained in a greenhouse in soil. The plants were watered regularly and when necessary, were sprayed to eliminate fungi and insects. Growth was encouraged by pruning and fertilization with a slow release fertilizer (OSMOCOAT®).

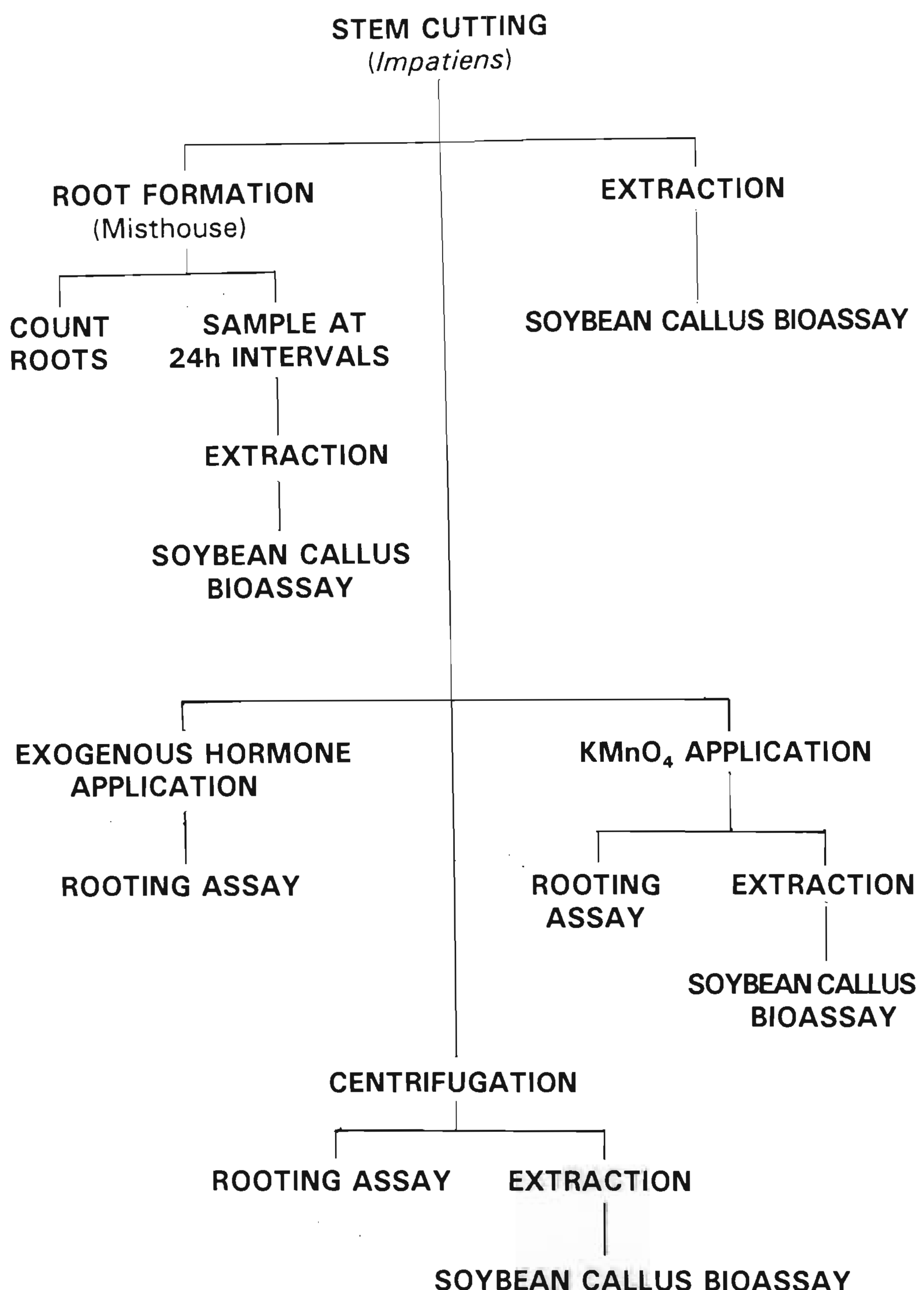


Figure 2.1 Flow diagram summarizing the experimental procedures applied to *Impatiens* stem cuttings.

Erythrina caffra Thunb. is an easily grown deciduous tree belonging to the family Fabaceae (Subfamily Faboideae). The genus is widespread in tropical and subtropical regions (PIENAAR, 1984). This tree was selected to compare against *Eucalyptus*, as it is fairly easy to root in the absence of any rooting hormones. Stock material was obtained from the Botany Department Gardens.

2.2 Rooting of cuttings (greenhouse)

Cuttings, standardised with respect to stem diameter and cut to a uniform length of 15 cm, were inserted to a depth of 20-30 mm in holes prepared in a vermiculite medium in speedling trays. The trays were placed in a misthouse under intermittent mist irrigation, the frequency of which was controlled automatically by an electronic leaf solenoid-valve system. The temperature varied from 22-26°C (mean air temperature 25°C; bed temperature 21°C). The mist setting ensured that the cuttings did not wilt and desiccate while rooting occurred.

2.3 Rooting assays

2.3.1 Mung Bean Rooting Bioassay

The mung bean (*Vigna radiata* (L.) Wilczek) rooting assay was used to detect root initiation. This assay was developed by HESS (1961c) for the detection of substances, other than IAA, stimulating root initiation.

Mung bean seeds were surface sterilized in a 3.5% sodium hypochlorite (JIK®) solution for 20 min before being rinsed thoroughly and soaked for 6 hours in distilled water. The seeds were planted in trays of moist vermiculite and germinated in a growth cabinet under continuous light (32.1 μmol m^{-2} s^{-1}) at 25°C.

After 9 days the hypocotyls, including two primary leaves, were cut to a length of 10 cm, and the cotyledons removed. These cuttings were transferred immediately to the test solutions and immersed to a depth of 5 cm. Two cuttings were placed in each vial. Five vials were used per treatment. The cuttings remained in the test solutions for 8 hours in the growth cabinet. After this period, the cuttings were rinsed and placed in vials containing distilled water. These were placed randomly in trays and returned to the growth cabinet for a period of 10 days. The number of roots and lateral roots produced on each cutting was subsequently recorded (CROUCH AND VAN STADEN, 1991).

2.3.2 Root Culture

A root culture suspension system, previously initiated from sterile *Lycopersicon esculentum* (tomato), roots was maintained on a rotary shaker (120 rpm) in continuous light (3.9 μmol m^{-2} s^{-1}) at 25°C. The root material was subcultured at 3-4 week intervals. Due to the initially slow rate of growth, the nutrient medium was changed from the original WHITE'S (1934) medium to full strength MILLER'S (1965) medium (excluding hormones) (TABLE 1). The stock roots were grown in a liquid medium with 2% sucrose (FINNIE AND VAN STADEN, 1985).

TABLE 1. Basal medium for soybean callus bioassay. (Adapted from MILLER, 1963, 1965).

STOCK	CHEMICALS	MASS IN STOCK ($g\ \ell^{-1}$)	VOLUME STOCK ADDED ($m\ell\ \ell^{-1}$)
1	KH_2PO_4	3.0	100
	KNO_3	10.0	
	NH_4NO_3	10.0	
	$Ca(NO_3)_2.4H_2O$	5.0	
	$MgSO_4.7H_2O$	0.715	
	KCl	0.65	
	$MnSO_4.4H_2O$	0.14	
2	NaFeEDTA	1.32	10
	$ZnSO_4.7H_2O$	0.38	
	H_3BO_3	0.16	
	KI	0.08	
	$Cu(NO_3)_2.3H_2O$	0.035	
	$(NH_4)Mo_7O_{24}.4H_2O$	0.01	
3	MYO-INOSITOL	10.0	10
	NICOTINIC ACID	0.2	
	PYRIDOXINE HCl	0.08	
	THIAMINE HCl	0.08	
4	NAA	0.2	10

2.3.3 Statistical Analysis

One-way analysis of variance and Tukey's range test were used in the statistical analysis of all data. The confidence level was set at 95%. All experiments were repeated at least twice.

2.4 Extraction Techniques for cytokinins (Refer to Fig.2.2)

The stem tissue was homogenised in 100 mℓ 80% (v/v) ethanol, and placed on a shaker for 12 hours at room temperature. The homogenate was then filtered under vacuum through Whatman No. 1 filter paper. The residue was washed with 10 mℓ 80% ethanol before being discarded. The filtrate was concentrated to the aqueous phase under vacuum at 35°C using a rotary evaporator (SMITH AND VAN STADEN, 1978). This constituted the crude extract.

2.5 Fractionation and Identification of Cytokinins (Refer to Fig.2.2)

The procedures used to isolate plant hormones are largely dependent on chromatographic techniques for purification of the extract. Crude extracts rarely give accurate indications of biological activity (HORGAN, 1981).

2.5.1 Column Chromatography

Ion exchange chromatography is valuable as an initial purification step. It provides a reliable method for the separation of cytokinin ribotides from the other forms of cytokinin present (HORGAN, 1981).

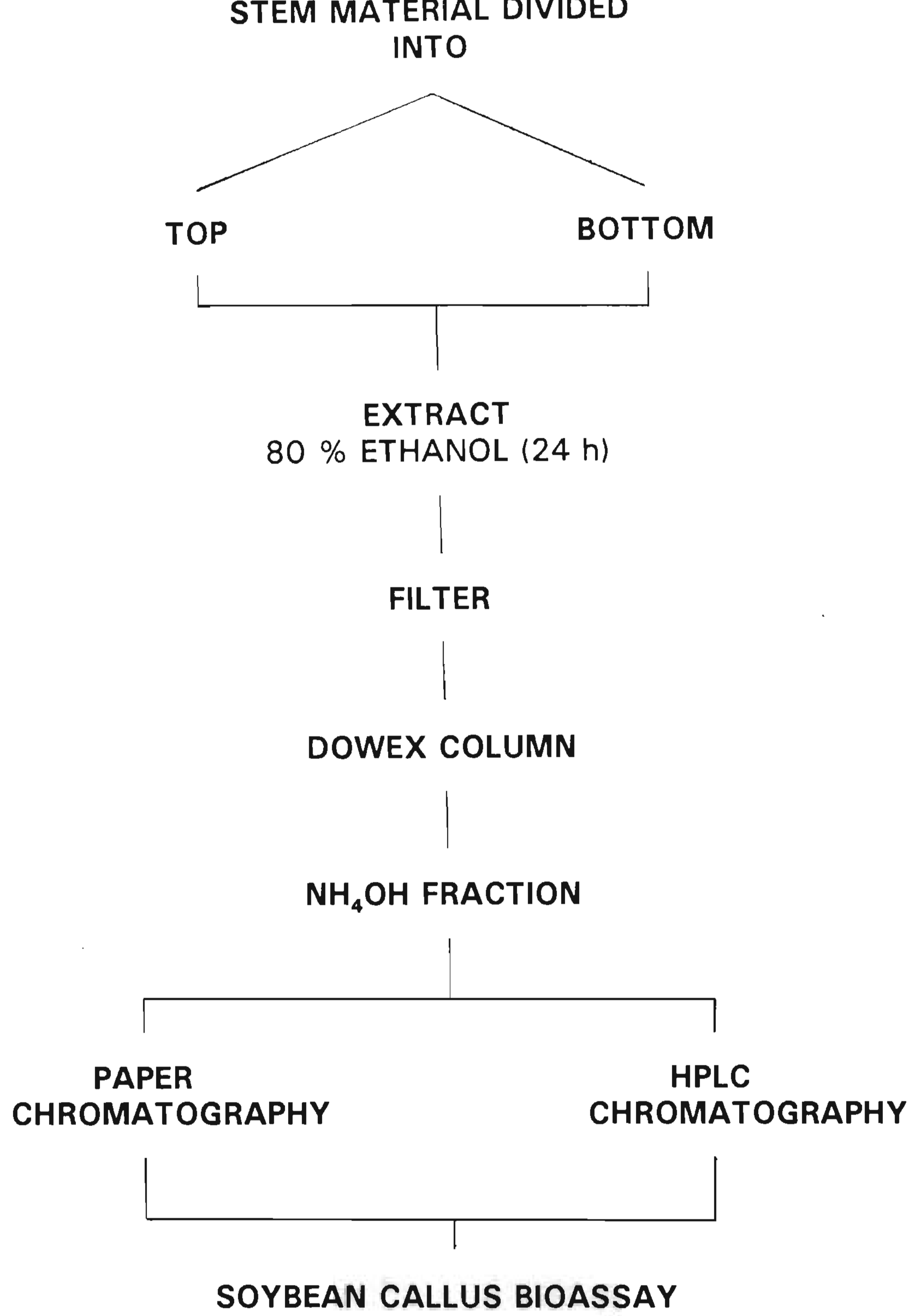


Figure 2.2 Flow diagram summarizing the extraction procedure for stem cuttings.

The crude extract was resuspended in 60% (v/v) ethanol and the pH was adjusted to 2.5 with 1 N HCl. This acidified extract was then passed through a Dowex 50W-X8 cation exchange resin (H+ form: 200-400 mesh, column 2x8 cm) at a flow rate of approximately 10 mℓ h^{-1}.The column was washed with 50 mℓ 80% ethanol and the adsorbed cytokinins were eluted with 100 mℓ 5N ammonium hydroxide. The ammonia fraction was collected and dried under vacuum at 35°C before resuspension and further analysis (VAN STADEN, 1976a).

2.5.2 Paper Chromatography

The vacuum-dried residues were redissolved in 90% (v/v) ethanol and applied as a 1 cm strip on Whatman No. 1 chromatography paper. The cytokinins were separated using descending chromatography with a solvent system of isopropanol:ammonia:water (10:1:1 v/v). The chromatograms were run to a length of 40 cm. They were dried in a stream of air, at 50°C, then divided into 10 R_f strips and assayed using the soybean callus bioassay (VAN STADEN, WEBB AND WAREING, 1972; SMITH AND VAN STADEN, 1978).

2.5.3 HPLC Separation of Extracts

In order to obtain more information about the nature of the cytokinins constituting the slow (polar) and fast moving (non-polar) peaks, the polar and non-polar parts of the paper chromatograms were eluted with 80% ethanol. Each eluate was then dried under vacuum and resuspended in 500 μℓ 80% methanol. The sample was filtered using a 0.22 μm Millepore filter before injection for HPLC. A Hypersil 5 ODS semi-preparative column (25 x 10 mm) was used for separation. UV detection was recorded at 254 nm using an absorption range of 0.05.

2.6 Soybean Callus Bioassay

Despite the rapid improvement of techniques used in the isolation, purification and identification of cytokinins, bioassays remain an integral part of the identification process. In addition most of the literature on endogenous cytokinins has been based on information obtained from bioassays (VAN STADEN AND DAVEY, 1979). To be strictly accurate, only compounds that have been chemically identified should be named cytokinins. Other compounds should be referred to as cell-division-inducing compounds, or cytokinin-like compounds, and should be qualified by reference to the bioassay used (VAN STADEN AND DAVEY, 1979). Initial studies conducted using bioassays can provide primary qualitative information on the nature of endogenous hormones in an extract (HORGAN, 1981). All cytokinin-like compounds described in this thesis have been tentatively classed by co-chromatography.

Callus from soybean cotyledons (*Glycine max* (L.) Merrill cv. Acme) is cytokinin-dependent and is capable of rapidly metabolising cytokinins (FORSYTH AND VAN STADEN, 1986). The bioassay exhibits a linear relationship between response and concentration over a wide range of cytokinin concentrations (VAN STADEN AND DAVEY, 1979). The callus had been initiated according to the procedure described by MILLER (1963, 1965) and was maintained on MILLER's medium (1965), supplemented with kinetin (0.5 mg ℓ^{-1}) and NAA (2 mg ℓ^{-1}) for numerous subcultures (TABLE 1) .

Agar (1%) was weighed into bioassay flasks containing the dry cytokinin samples. MILLER's medium (30 mℓ in 50 mℓ flasks and 15 mℓ in 25 mℓ flasks) was dispensed into each flask (TABLE 1). The flasks were stoppered with non-absorbent cotton wool bungs and covered with aluminium foil. The flasks and transfer instruments were autoclaved at 121°C and 1 Pa for 20 min. The flasks were transferred to a sterile cabinet and the agar allowed to set under UV light. Three small pieces of callus (approximately 10 mg) were transferred

to each flask. The flasks were placed in a growth room with constant temperature (26°C ± 2°C) and continuous, low light intensity (0.72 μmol m^{-2} s^{-1}) supplied by cool white fluorescent tubes, for 28 days. The combined mass of the three callus pieces was recorded as callus yield (VAN STADEN, 1976b).

2.7 Problems Associated with Experimental Techniques

It should be noted that it is virtually impossible to conduct ideal experiments with intact plants since the manipulation of whole plants is, at best, disruptive. Specifically, the severing of a plant, or plant part, will inevitably produce a wound response and will interrupt the hormonal signals normally received by the intact plant. Substituting various applied hormones for the plant parts could lead to the presence of hormones in unnatural places and to the unusual metabolism of these hormones, both at the cut surface and within the plant. In addition, exogenously applied hormones may increase an endogenous pool to the point of producing hormone imbalance and metabolic disturbance (MATTHYSSE AND SCOTT, 1984).

An increasingly important consideration is the unequivocal identification and quantification of hormones extracted from plants. Clearly, to determine when a hormone is performing as a hormone, it is necessary to distinguish active, accessible; and inactive, less accessible, or stored forms of the hormone. Long term experiments require careful analysis of processes and kinetics involved, and the possibility of any intermediate steps. The presence of microorganisms as contaminants must be avoided since they are likely to affect both long and short term processes (MATTHYSSE AND SCOTT, 1984).

CHAPTER 3

ANALYSIS OF STEM CUTTINGS TO DETERMINE THE CYTOKININ CONTENT

3.1 Introduction

Roots form readily on cuttings of many herbaceous species without the need for special treatments. This is especially true for stem cuttings, although in many plants the leaves and cotyledons may also root vigorously (LOVELL AND WHITE, 1986).

Previous investigation into the activity of endogenous cytokinins in cuttings with respect to rooting was conducted on two species of *Populus* - the difficult-to-root *P. euramericana* and the easy-to-root *P.tremula*. Higher levels of cytokinin activity, at various stages of root formation, were observed in cuttings of the species that was hard to root. (OKORO AND GRACE, 1978).

Studies conducted on leaf cuttings of *Phaseolus vulgaris* indicated both quantitative and qualitative differences in the types of cytokinins present in different aged leaves. The activity detected in 10-day-old leaves co-chromatographed with zeatin/zeatin riboside. This showed as a much smaller peak in 30-day-old leaves, which had additional activity corresponding to zeatin glucoside, and possibly dihydrozeatin-O-glucoside. There was a correlation between the initial cytokinin content of the leaves and their rooting ability. The roots produced by the 10-day-old leaf cuttings were fewer, longer and thicker, with a higher content of cytokinin activity than the many fibrous roots produced in the 30-day-old leaf cuttings (FEATONBY-SMITH AND VAN STADEN, 1981).

The following experiments were initiated to determine the normal levels and types of cytokinins present in the respective plants. A comparison of the stem material of both easy- and difficult-to-root plants was conducted in order to ascertain whether any differences in rooting ability could be correlated with different cytokinin levels. The experiment was further designed to determine whether cytokinin activity differs between the upper and lower portions of the stem.

3.2 Materials and methods

Two sets of cuttings of each species under investigation were collected. One set of cuttings was allowed to root in the misthouse (Chapter 2), while the second batch was set aside for extraction. Stem cuttings approximately 10 cm long were collected and immediately divided into equal upper and lower portions. The leaves and, if present, flowers were removed prior to extraction.

The stem material was weighed and then cut into small pieces before homogenisation in a Waring blender with 100 mℓ 80% ethanol. The extraction method described in Chapter 2 was followed.

An initial set of extracts was run on paper (Chapter 2) and then assayed using the soybean callus bioassay. This procedure was followed for the *Impatiens*, *Vigna radiata*, *Erythrina*, and the three *Eucalyptus* species (*E.grandis*, *E.macarthuri*, and *E.nitens*). The results are presented in Figures 3.1 and 3.2. Further extracts were prepared for the *Impatiens*, *Vigna radiata*, and the *Eucalyptus* species. These were run through a HPLC semi-preparative column and the ninety fractions collected were assayed with the soybean callus bioassay (Chapter 2). These results are presented in Figures 3.3 - 3.5. In each figure, the horizontal line represents the control, or zero cytokinin growth response of the callus. The results are discussed with reference to the

standards run for each experiment. A comparison across experiments was also made.

3.3 Results

The results obtained for the three species classified as "easy-to-root" are presented in Figure 3.1 A-C. In all three cases a range of cytokinin-like activity was detected. Activity was observed in the fast-moving non-polar fractions (R_f 0.6-1.0). Peaks co-chromatographing with zeatin and zeatin riboside were observed. When this activity is correlated to that of the kinetin standards, it is evident that the *Impatiens* extracts had a very low cytokinin activity (Fig.3.1 A) - approximately equivalent to 10 $\mu g/\ell$ kinetin, while the *Vigna radiata* cuttings (Fig. 3.1 B) yielded activity greater than the 50 $\mu g/\ell$ kinetin standard. The activity in the *Erythrina* cuttings falls between the 10 and 50 $\mu g/\ell$ standards (Fig. 3.1 C). The upper halves of the mung bean cuttings contained in addition, a slow-moving peak, co-eluting in the region of zeatin-glucosides (Fig. 3.1 B).

Both the *Impatiens* and the *Vigna radiata* cuttings produced roots within 10 days. The *Erythrina* cuttings produced roots after 4 weeks.

The results for the three *Eucalyptus* species are presented in Figure 3.2 A-C. The peaks of cytokinin-like activity extracted from these plants were much sharper than those illustrated in Figure 3.1. There was a clear similarity in the types of cytokinins extracted from all three *Eucalyptus* species, with those cytokinins co-chromatographing at R_f 0.7; 0.8 and 1.0 in the upper half, and R_f 0.6 and 0.7 in the lower half common to all three.

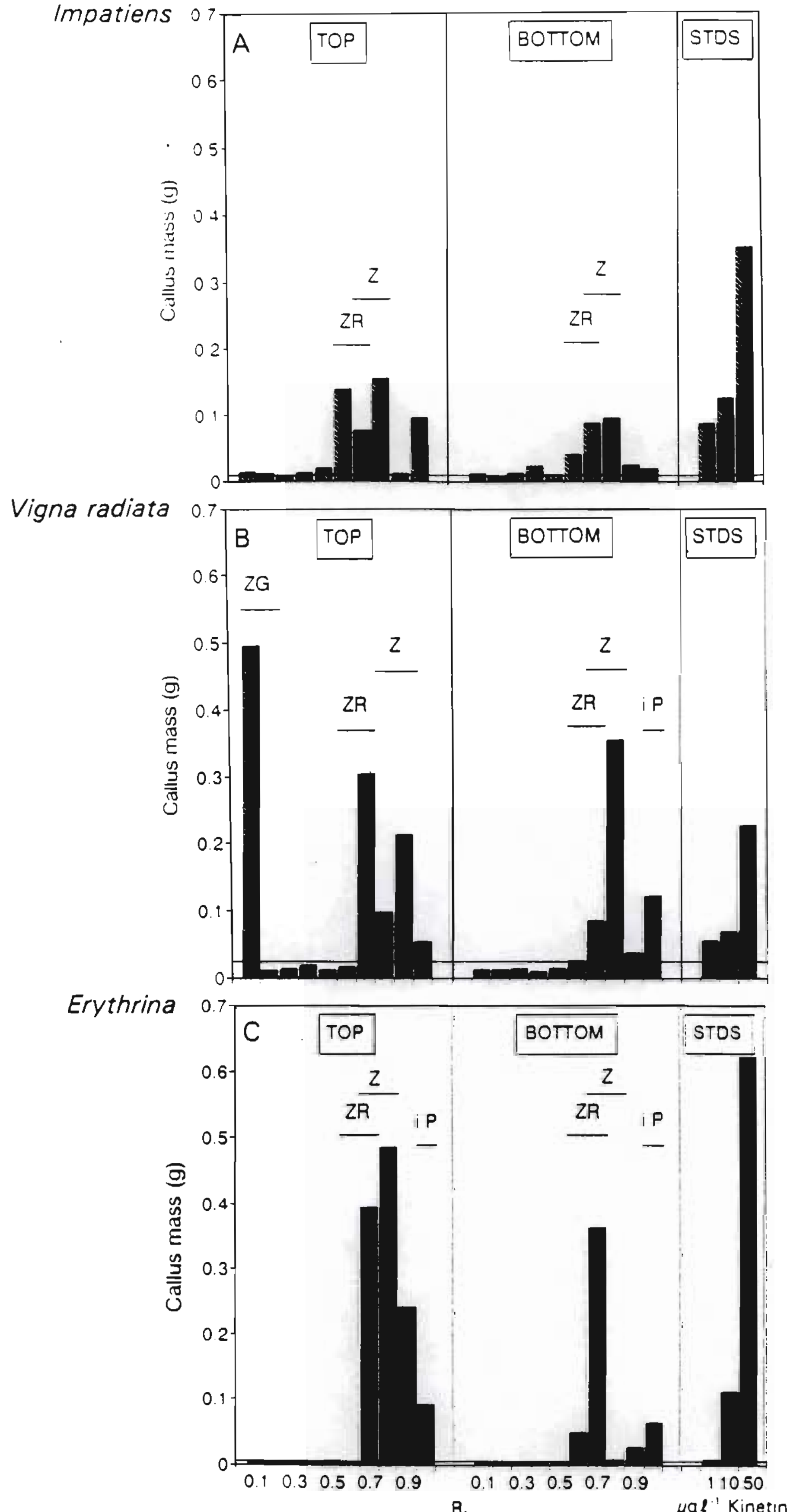


Figure 3.1 Soybean callus bioassays of extracts of stem material from (A) *Impatiens*; (B) *Vigna radiata;* (C) *Erythrina*; separated by paper chromatography.

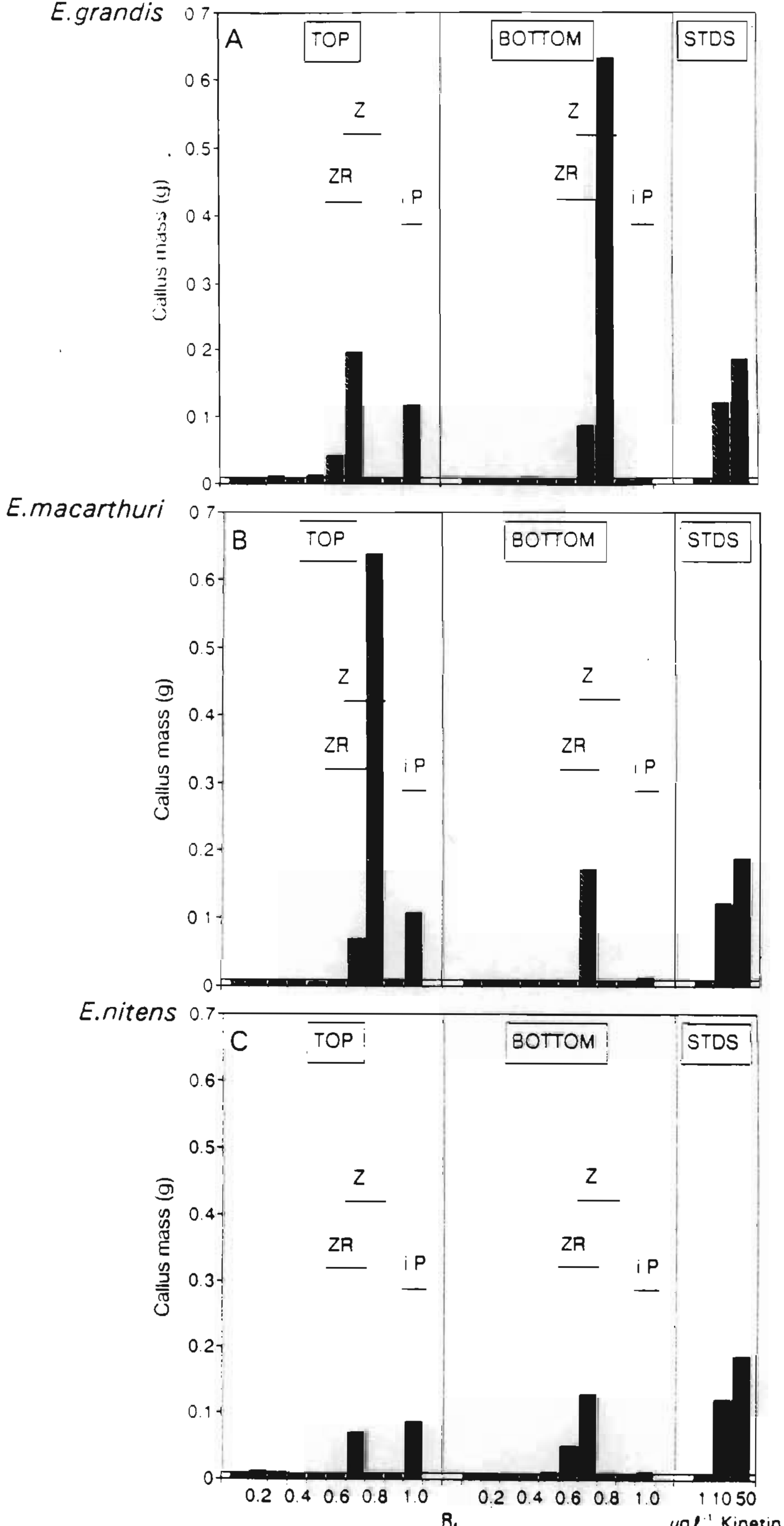


Figure 3.2 Soybean callus bioassays of extracts of stem material from (A) *Eucalyptus grandis*; (B) *E.macarthuri*; (C) *E.nitens*; separated by paper chromatography.

E.grandis and *E.macarthuri* were the only two *Eucalyptus* species to produce roots. This occurred after approximately 12-16 weeks, without the addition of rooting hormone (IBA powder), and after 8-12 weeks with the addition of IBA. *E.nitens* did not produce roots, and the cuttings senesced and died.

Figures 3.3-3.5 represent the results of the HPLC separation of the respective extracts. The results for the *Impatiens* separation show two peaks of activity in the upper stem half and three areas of activity in the lower half. These peaks coeluted with the standards zeatin, dihydrozeatin, dihydrozeatin riboside and isopentenyladenine (Fig. 3.3 A), and zeatin and dihydrozeatin (Fig. 3.3 B).

The results obtained for *Vigna radiata* show activity in only one of the fractions collected from the upper half of the stem. In the lower half, two small peaks of activity were recorded in the region of zeatin/dihydrozeatin and isopentenyladenine (Fig. 3.4 A&B).

Very little activity was detected in the *E.grandis* extracts (Fig. 3.5 A&B). Only two small peaks, in fractions 36 and 55, were recorded for the extract from the lower half of the cuttings (Fig. 3.5 B). In contrast, both *E.macarthuri* and *E.nitens*, yielded cytokinin-like activity over most of the fractions collected (Fig. 3.5 C&D and Fig. 3.5 E&F). In both instances the activity observed in the lower half of the stem was considerably less than in the upper half (Fig. 3.5 D and Fig. 3.5 F).

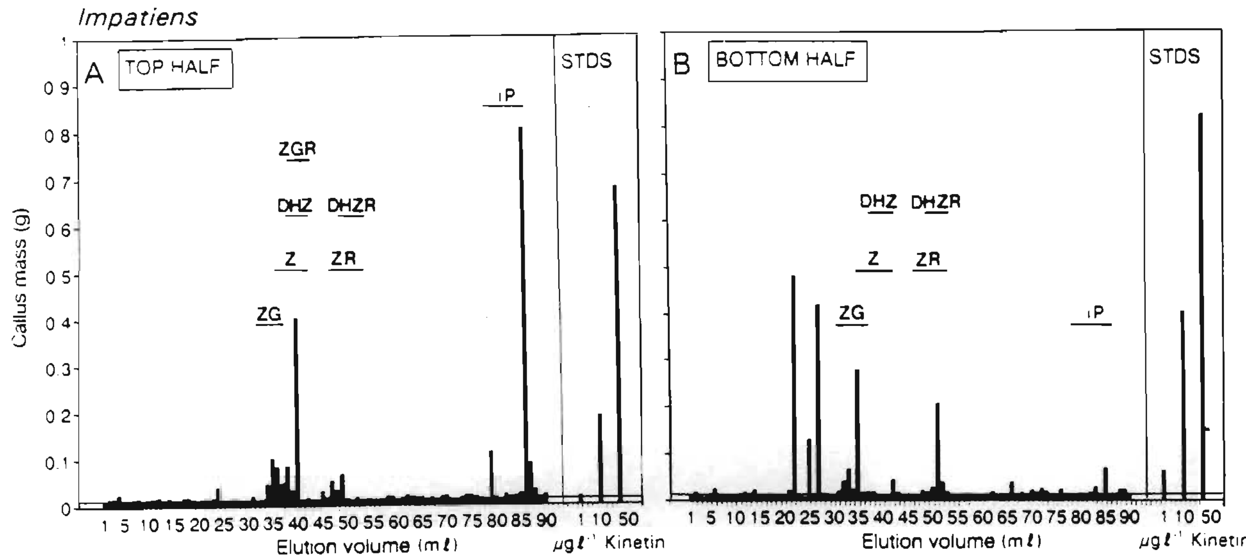


Figure 3.3 Soybean callus bioassays of extracts of *Impatiens* stem material (A) top half; (B) bottom half of cuttings; separated by HPLC.

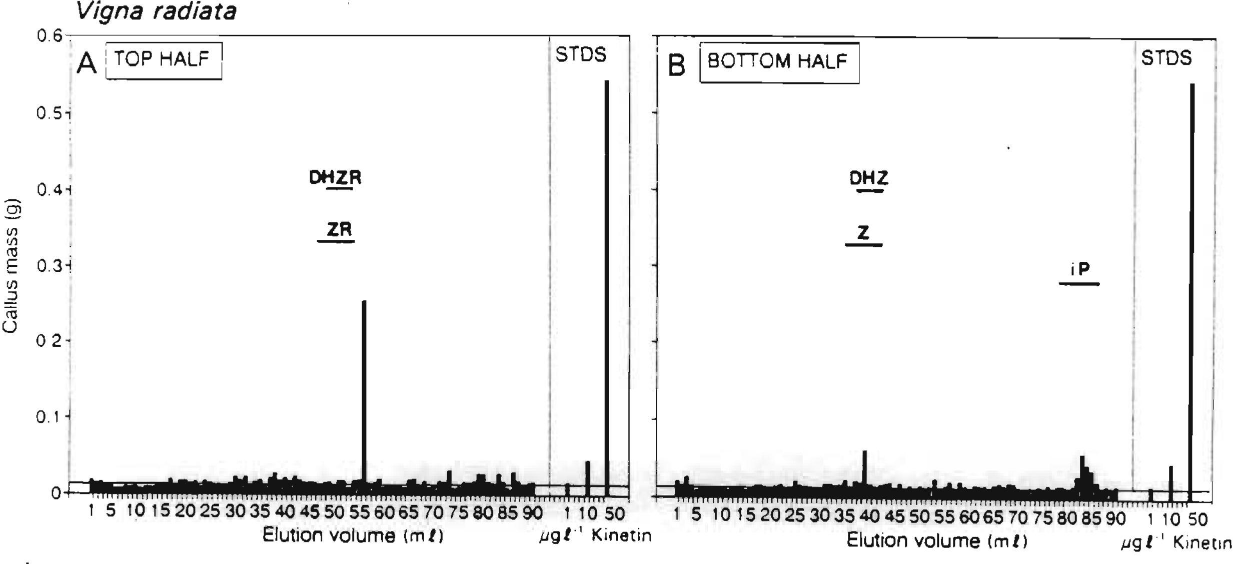


Figure 3.4 Soybean callus bioassays of extracts of *Vigna radiata* stem material (A) top half; (B) bottom half of cuttings; separated by HPLC.

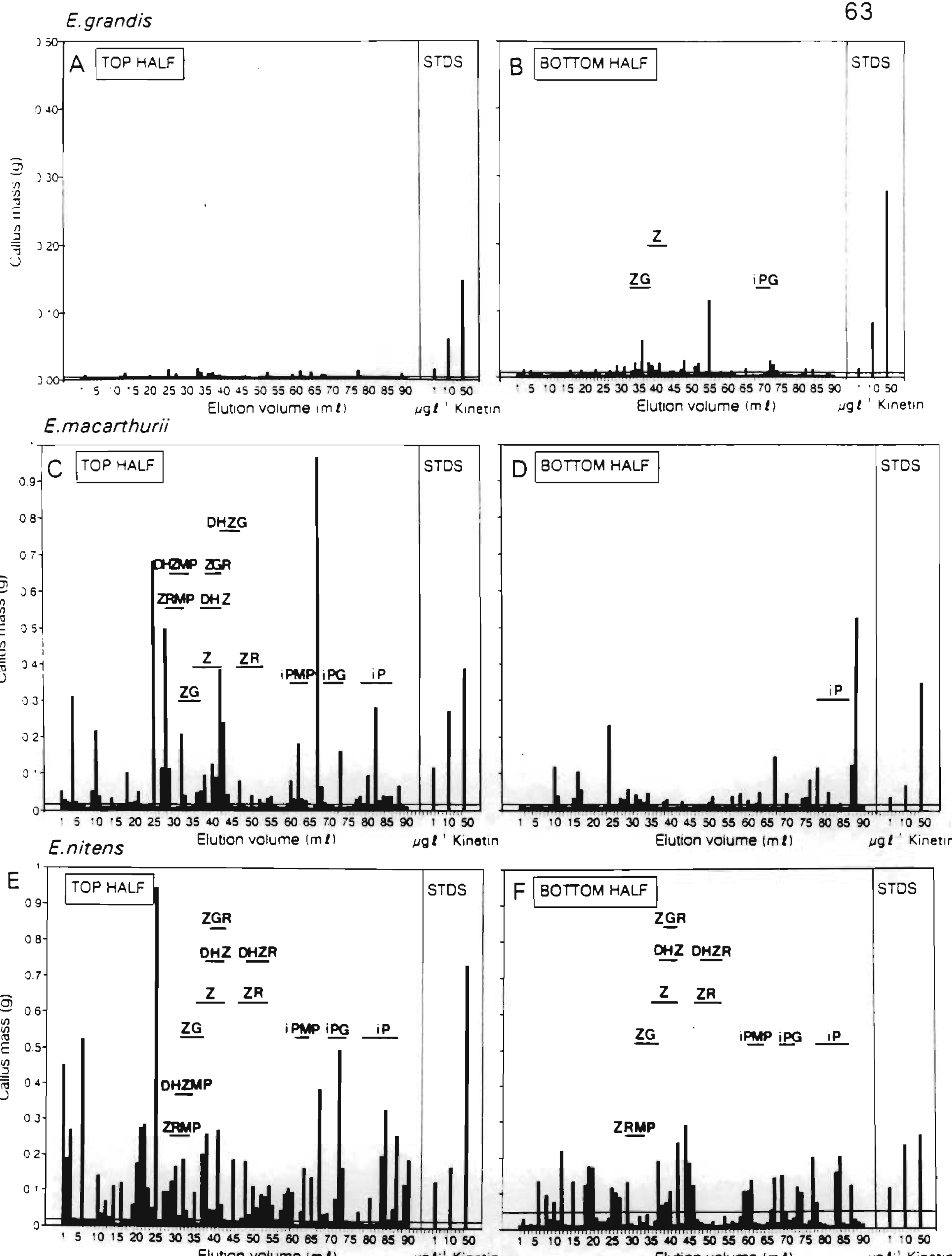


Figure 3.5 Soybean callus bioassays of extracts of *Eucalyptus* stem material (A) *E.grandis* top half; (B) *E.grandis* bottom half of cuttings; (C) *E.macarthuri* top half; (D) *E.macarthuri* bottom half of cuttings; (E) *E.nitens* top half; (F) *E.nitens* bottom half of cuttings. Extracts separated by HPLC.

3.4 Discussion

A comparison of the cytokinin levels in the "easy-" and "difficult-to-root" plants does not show a distinctive trend in the levels or types of these hormones. Qualitatively the activity in each of the extracts is similar, with cytokinin-like activity detected at the elution volumes corresponding with zeatin and zeatin riboside.

Only *Vigna radiata* yielded an additional peak of activity at R_f 0.1 (which could correspond to zeatin glucoside). These results are representative of the ammonia fraction collected from the Dowex column, and will include any cytokinin bases, ribosides and glucosides present in the sample. The ethanolic fraction, in which the nucleotides elute, contained too high a proportion of impurities to allow successful chromatography and analysis with the soybean callus bioassay.

In *Phaseolus vulgaris*, polar and non-polar peaks of activity were isolated (BRIDGLALL AND VAN STADEN, 1985). These peaks were found to co-chromatograph with zeatin glucoside, and zeatin and zeatin riboside respectively. These compounds co-chromatographed on paper with their dihydro-derivatives (BRIDGLALL AND VAN STADEN, 1985). This raises the possibility that these cytokinins are also present in the material under investigation.

It is interesting to note that BRIDGLALL AND VAN STADEN (1985) found that in material treated with auxin (5 and 20 $mg\ell^{-1}$ IBA for 20 h), this polar peak (R_f 0.1-0.2) was missing.

The activity detected in the *Eucalyptus* cuttings co-chromatographed with the zeatin and zeatin riboside standards. The levels of activity in these cuttings were higher than those recorded for the kinetin standards. While this seems to indicate both a quantitatively higher proportion of cytokinin-like

compounds, it must be remembered that naturally occurring cytokinins do show relatively higher levels of activity in bioassays than do the synthetic ones (LETHAM, 1978). Specifically, in the soybean callus bioassay, the following order of effectiveness has been observed: DHZ-OG > Z >> K; Z > ZR > Z-OG; BA > K > iP; acyl substituted N-6-adenines and adenine at high concentrations are active in the assay (YOPP, AUNG AND STEFFENS, 1986).

Previous research has shown that qualitatively, the cytokinins of both the difficult-to-root ***Populus tremula*** and the easy-to-root ***P.euramericana*** appear identical on the basis of chromatographic behaviour (OKORO AND GRACE, 1978). The persistently high levels of cytokinins in the difficult-to-root species (*P.tremula*) could have contributed to its inability to root, as it is known that exogenously applied cytokinins inhibit root initiation in cuttings (OKORO AND GRACE, 1978).

Endogenous rooting inhibitors may have an overriding influence on adventitious root formation in adult tissues of *E.grandis* (NICHOLLS, CROW AND PATON, 1972). It was suggested that a relationship exists between the decreased rooting ability of stem cuttings and increased levels of a rooting inhibitor in the tissue at the base of the cutting. This inhibitor was found to decompose in water. The high minimal concentration required for activity in bioassays (10^{-4}M) implies that the physiological basis of the activity of this inhibitor may differ from that found for the typical hormonal regulation where activity is often detected at much lower concentrations (natural concentrations in plants being in the region of 2 x 10^{-3} M). Three closely related inhibitory compounds were isolated - each inhibited the rooting of stem cuttings of ***E.grandis*** seedlings, ***E.deglupta*** seedlings and mung bean seedlings at 10^{-4}M (NICHOLLS, CROW AND PATON, 1972).

These results indicate a similar trend in the qualitative detection of the cytokinin-like activity in the cuttings. It is thus possible that it is not the initial

levels of cytokinin that are important in the initiation of rooting, but the ability of the plant tissues to metabolise these cytokinin-like compounds. KEFELI (1978) reported that auxins are decomposed in the course of rooting, i.e. the rooting of cuttings is accompanied by changes in the endogenous auxin content. Furthermore, the activity of natural inhibitors, which interfere with the rhizogenesis process, also changes. These changes are, however, not as easily predicted as for auxins. It was demonstrated that the auxin and growth inhibitor contents remain essentially the same during the rooting of difficult-to-root species whereas activity of these groups of substances undergoes radical changes in easily rooting species (KEFELI, 1978).

The results presented in this investigation show that qualitatively, the cytokinin content of the upper and lower stem sections is similar. Quantitatively there was not, as was expected, a higher proportion of these cytokinins in the lower section. There was thus no evidence of a cytokinin gradient in the stem. In most instances the higher cytokinin-like activity was observed in the upper portion.

The relatively higher cytokinin-like activity in the *Eucalyptus* species could explain the longer period required for root development. These high levels would take longer to be metabolised to inactive forms. The low levels and limited types of cytokinin-like activity in the extracts from *E.nitens*, when related to the inability of this species to produce roots, seems to contradict this. It is thus probable that endogenous rooting inhibitors play a role in preventing root initiation (NICHOLLS, CROW AND PATON, 1972). In addition, the multitude of other factors described in Chapter 1 cannot be ignored.

Previous investigations have revealed the necessity of a minimal cytokinin content for the efficient rooting of cuttings (ERIKSEN, 1974; BATTEN AND GOODWIN, 1978). It was proposed by BATTEN AND GOODWIN (1978) that those cuttings that do not respond to the application of auxin (e.g. *E.nitens*), lack sufficient levels of endogenous cytokinins to produce adventitious roots.

CHAPTER 4

A CORRELATION BETWEEN STAGE OF ROOT DEVELOPMENT AND CYTOKININ LEVELS AND TYPES

4.1 Introduction

The results described in the previous Chapter suggest that while qualitatively and quantitatively the cytokinin content of cuttings may be similar, and cannot be dismissed as unimportant, it may be the stem tissues' ability to alter these levels through metabolism that enables efficient adventitious root initiation and development. The cytokinin content of cuttings changes during the period of initiation and development of adventitious roots (ERIKSEN, 1974; OKORO AND GRACE, 1978).

It is probable that when cuttings are rooting their cytokinin content is gradually metabolised, in favour of bud and latent root growth, in the delay of senescence, or simply inactivation by plant tissues. This decline is checked and reversed when roots form. The *de novo* initiation of roots could take advantage of the period of low cytokinin content thus created, while restoring the normal cytokinin level when properly formed (OKORO AND GRACE, 1978).

The necessity for investigation into the age at which root primordia, within roots or stems, initiate the synthesis of cytokinins for translocation to the rest of the plant has been raised by VAN STADEN AND DAVEY (1979) and VAN STADEN AND HARTY (1988). With respect to germinating seeds, the young radical initially does not seem to be capable of synthesis. No activity could be detected in excised roots of maize within the first seven days of culture. The initiation of synthesis could be determined by the extent of cytokinins stored

in the kernel or the age of the root (VAN STADEN AND DAVEY, 1979). The possibility that the type of cytokinin present, or at least the ratio of individual cytokinins, changes with the initiation and development of the root primordia, is suggested by the results obtained from the experiments using applied cytokinins - specifically the changing effect of BA on pea cuttings of differing age (HARTMANN AND KESTER, 1983; DREWES AND VAN STADEN, 1989).

It was therefore decided to prepare a set of *Impatiens* cuttings and harvest a portion each day, over the period taken for the cuttings to produce roots. The basal 3 cm of the stem, where root primordia first develop was collected. A cutting took approximately 15 days for roots to emerge and grow to a length exceeding 1 cm.

4.2 Materials and methods

Uniform *Impatiens* stem cuttings were selected and placed in a vermiculite medium in speedling trays in a misthouse. Three cuttings were removed at 24 hour intervals and the basal 3 cm of the stem was retained for analysis.

The material was rinsed in water, weighed, and homogenised. Extraction was conducted overnight in 50 mℓ 80% ethanol. The extraction procedure described in Chapter 2 was followed. The crude extract was then streaked on half a sheet of paper as described earlier (Chapter 2.5.2).

The 10 R_f fractions were assayed using the soybean callus bioassay (Chapter 2.6). Where further HPLC analysis was conducted, a full sheet of paper was loaded and run, with half the paper used directly in the assay, and half separated first using HPLC to obtain 90 fractions before assaying. This was done by eluting the combined 10 R_f fractions overnight in 80% ethanol. The extract was then dried under vacuum and resuspended in 300 $\mu\ell$ 80% methanol. This was filtered through a 0.22 μm filter fitted to a syringe. This

extract was then applied to a HPLC semi-preparative column for fractionation.

4.3 Results

The data from each of the paper R_f fractions was plotted over the 15- day rooting period. Figure 4.1 A represents the slow-moving polar compounds, while Figure 4.1 B represents the non-polar compounds. The levels of polar, cytokinin-like activity, eluting at R_fs 0.1 to 0.5, remained consistently low (insignificant) until Day 7 (Fig.4.1 A). At this stage root primordia became visible for the first time.

The non-polar cytokinins gradually decreased until Day 3. At Day 6 there was a distinct increase in the activity at R_f 0.9. This was followed on Days 7-11 by an increase in the activity at R_fs 0.6, 0.7 and 0.8 (Fig.4.1 B). These levels remained fairly high from this point of development onwards.

A more detailed presentation of these results is shown in Figure 4.2 and Figure 4.3. Generally, in all five of the experiments conducted, root primordia were first visible on Days 6-9 (often visible through the epidermis or after sectioning). Some cuttings produced callus just prior to, and simultaneously with these primordia. From Day 8 onwards, the root primordia had penetrated the epidermis and had begun to function as roots.

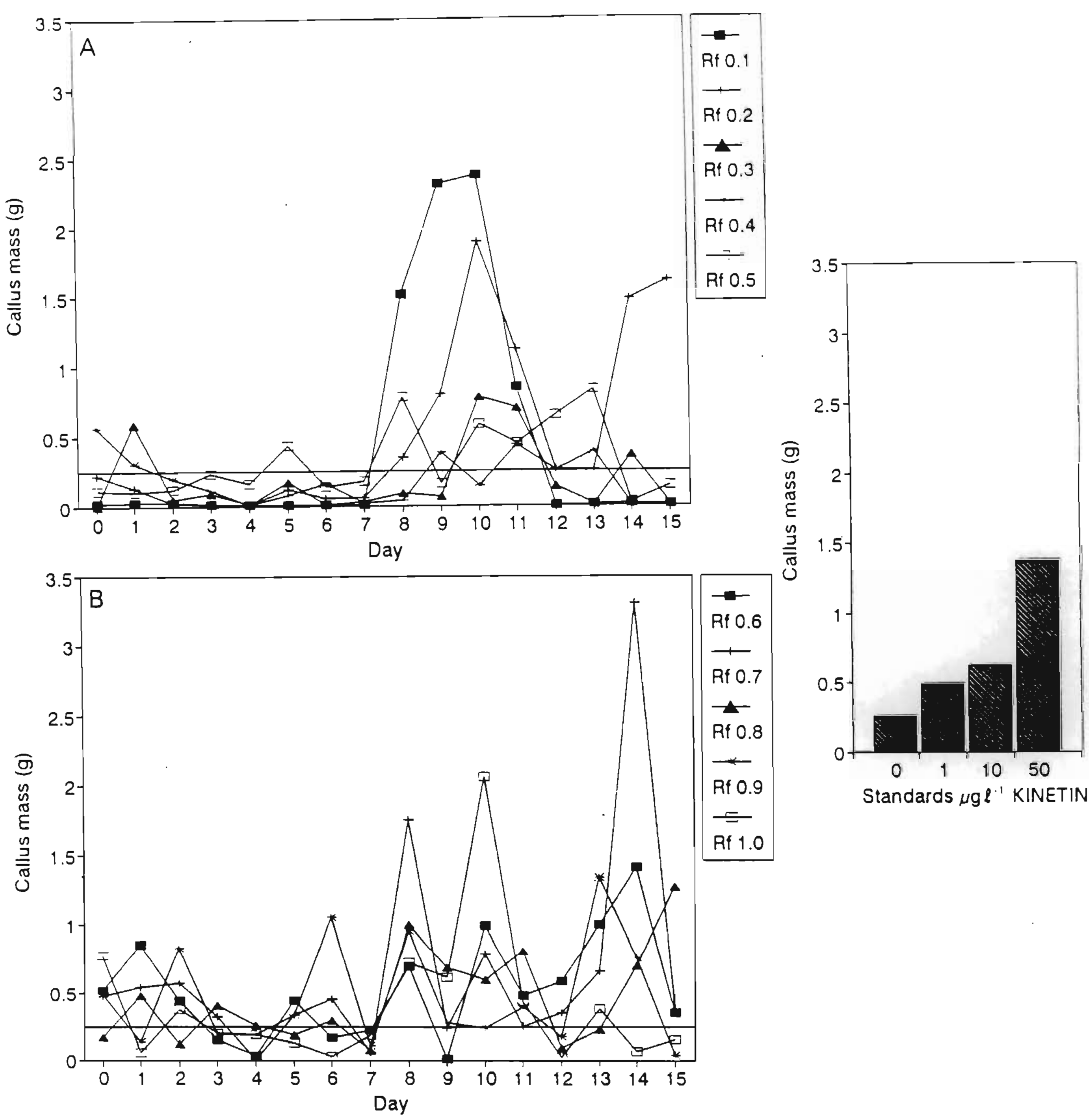


Figure 4.1 Soybean callus bioassay of extracts from the base of *Impatiens* stem cuttings harvested at 24 h intervals over the rooting period, and separated by paper chromatography. (A) polar cytokinin-like activity (R_fs 0.1-0.); (B) non-polar cytokinin-like activity (R_fs 0.6-1.0).

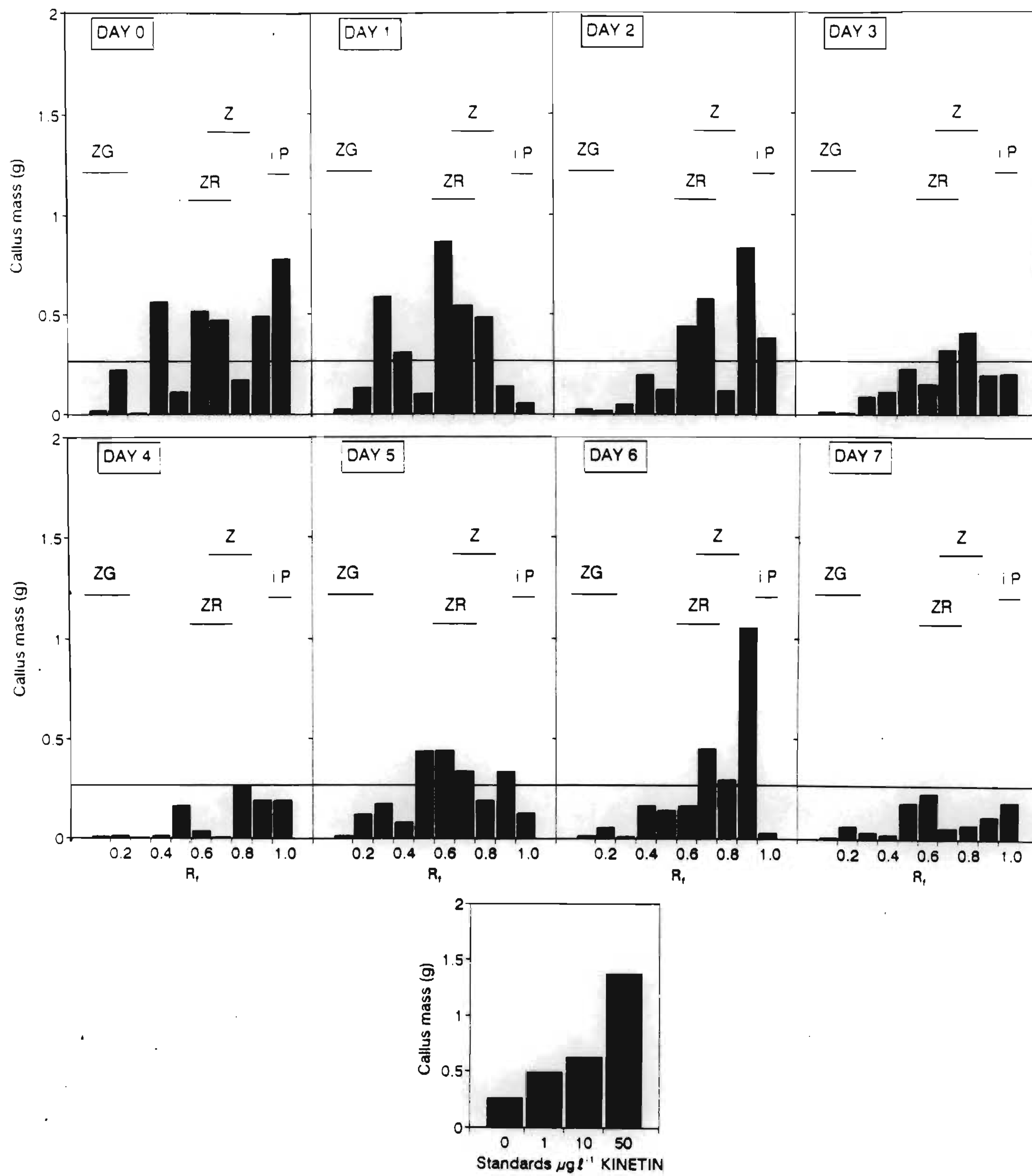


Figure 4.2 Soybean callus bioassays of extracts from the base of *Impatiens* stem cuttings harvested at 24 h intervals over the rooting period, and separated by paper chromatography (DAYS 0 - 7).

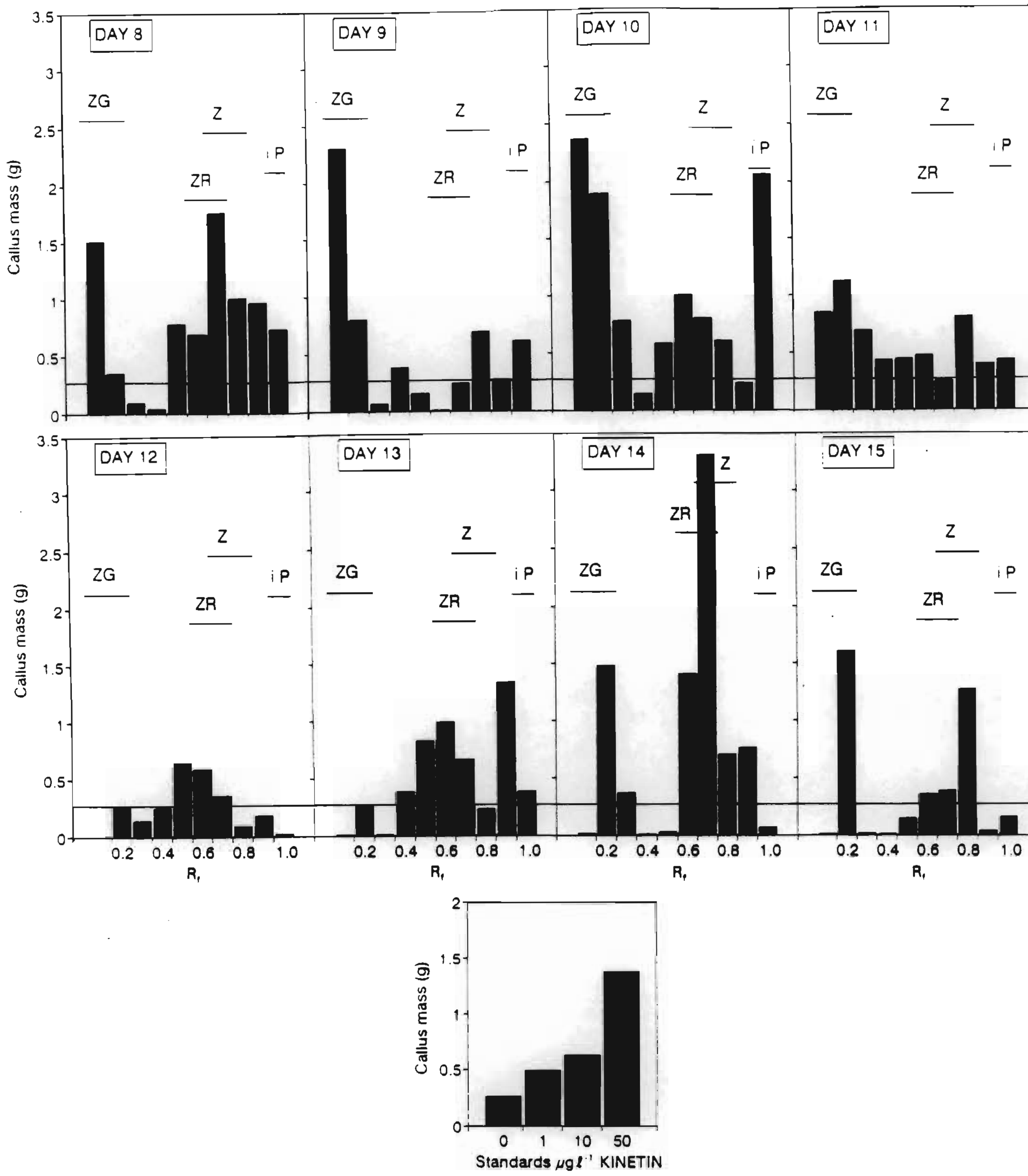


Figure 4.3 Soybean callus bioassays of extracts from the base of *Impatiens* stem cuttings harvested at 24 h intervals over the rooting period, and separated by paper chromatography (DAYS 8 -15).

Six of the extracts (Days 0, 2, 5, 6, 11 and 14) were fractionated using HPLC. The results of the soybean callus bioassays of these fractions are presented in Figure 4.4. Qualitatively, the initial (Day 0) cytokinin-like activity was similar to that observed for Day 14, indicating that the cycle of regeneration had neared completion. The original decrease in cytokinin-like activity is illustrated on Day 2 (Fig. 4.4). Cytokinin-like activity increased (Days 5&6) before the emergence of roots (Fig. 4.4) as was evident in Figure 4.1 B.

4.4 Discussion

Adventitious root initiation and development can be divided into three phases (KEFELI, 1978; JARVIS, 1986; WIESMAN AND RIOV, 1994). The first phase persists until the first cell divisions (0 - 2 days). During this phase there is increased sensitivity to IAA (inducing hormone), the decomposition of phytohormones (IAA) with participation of natural inhibitors, and the first cell divisions (KEFELI, 1978).

In *Impatiens* stem cuttings a decline in the cytokinin content of the base of the cutting was observed (Fig.4.1 A&B). Although *Populus* species activate preformed root initials as a response to wounding, there is, in addition, *de novo* synthesis of wound roots (OKORO AND GRACE, 1978), and so some comparison can be drawn. Investigation into the rooting of *Populus* cuttings showed an overall gradual decline in cytokinin levels observed in both the easy- and difficult-to-root species (OKORO AND GRACE, 1978). This general initial decrease in cytokinin activity was detected in both the upper and lower halves of the cuttings. This was suggested to indicate that the cytokinins underwent inactivation by plant tissues or were associated with bud activity.

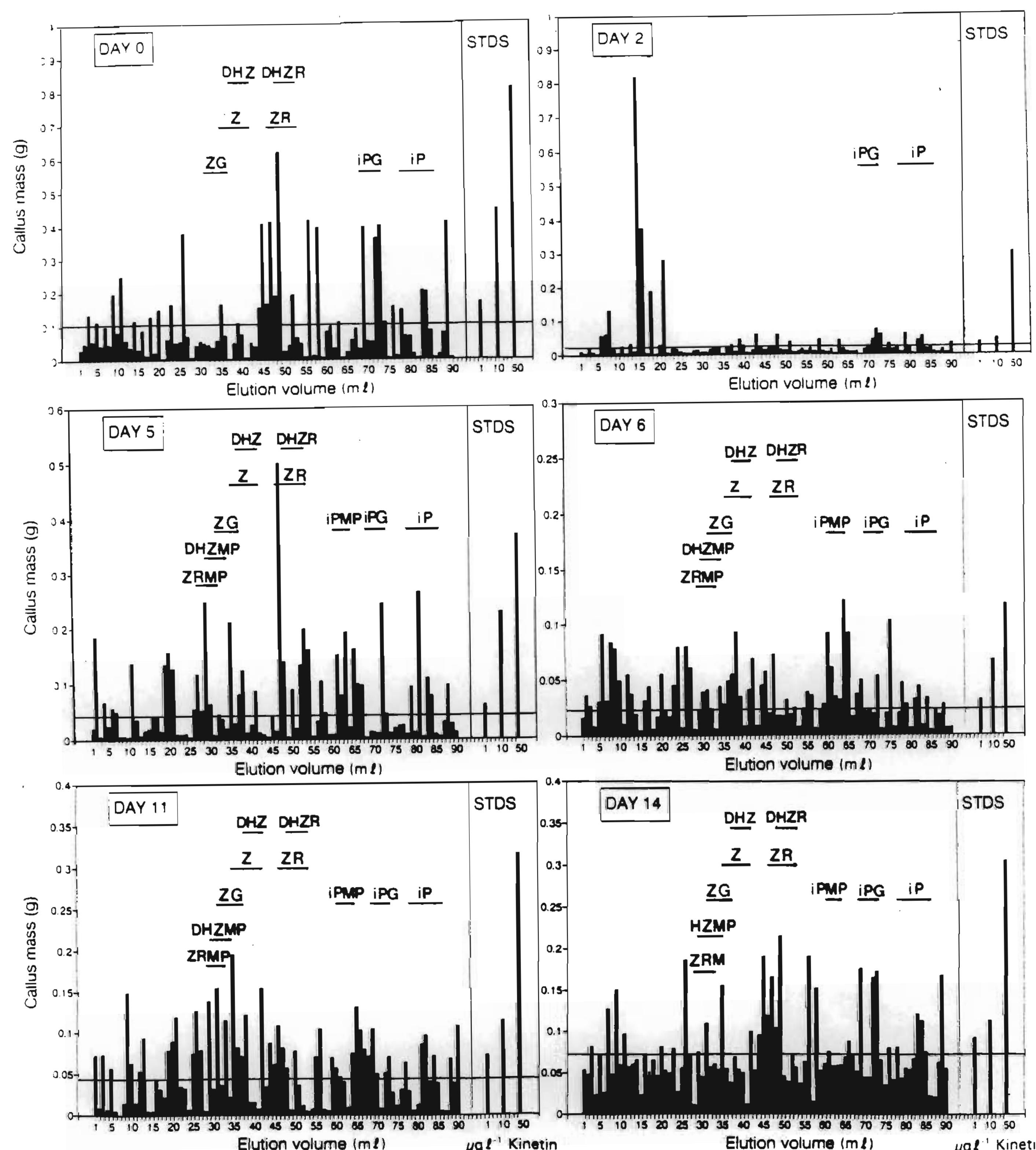


Figure 4.4 Soybean callus bioassays extracts from the base *Impatiens* stem cuttings, separated by HPLC. (Day 0; Day 2; Day 5; Day 6; Day 11; Day 14).

It was postulated that the cytokinins were converted to cytokinin glucosides, the storage form, which remained in the untested aqueous phase (OKORO AND GRACE, 1978). Similar decreases in endogenous cytokinin levels were noted for stem cuttings of *Pisum sativum* after the roots had been detached, indicating the importance of the roots in maintaining the supply of cytokinins to the stem (BOLLMARK, KUBAT AND ELIASSON, 1988).

The results presented in Figure 4.1 A show that both the polar (including cytokinin glucosides) fractions show a similarly sharp decline as the non-polar fractions (Fig. 4.1 B), followed by a period where they are absent from the stem extracts. The cytokinins must, therefore, either be utilized in the initial period of metabolic activity (cell division), or undergo conversion to a stable, inactive form. The low levels of cytokinins that occurred from Days 2 - 7 might have influenced the *de novo* initiation of wound roots (OKORO AND GRACE, 1978). A high cytokinin level inhibits root initiation at early stages of root development (ERIKSEN, 1974).

These levels remained consistently low for the following 2 - 3 days, i.e. Days 3 - 6 (Fig. 4.1). This is the period of radicle induction, and is characterised by active cell division, increased sensitivity to metabolic inhibitors, and a lack of response to phytohormones (IAA) (KEFELI, 1978). Root primordia were detectable from Day 6 onwards.

The final phase (Days 7-9) results in the formation of a root. This stage is characterised by the retardation of rhizogenesis, the elongation of the root, diminished sensitivity to inhibitors, and a lack of response to phytohormones (IAA) (KEFELI, 1978). Figure 4.1 A indicates a substantial increase in the cytokinin content of the stem after Day 7. These are polar compounds with activity at R_fs 0.1 and 0.2 particularly, co-chromatographing with cytokinin glucosides and monophosphates (these samples were not passed through a Dowex column which leads to the loss of cytokinin monophosphates). The non-polar compounds exhibiting activity in this bioassay, showed greater

fluctuations. This could be due to different rates of root initiation and development in the cuttings sampled on sequential days, despite all attempts to standardize the procedure. BOLLMARK, KUBAT AND ELIASSON (1988) found that, *Pisum sativum* stem cuttings, IPA levels increased earlier, and more gradually than ZR levels. In Figure 4.1 B, compounds co-chromatographing with iP showed an increase in activity earlier, but also more fluctuations than obseved for Z and ZR (which showed a gradual increase). ERIKSEN (1974) suggested that cytokinin enhances development of root primordia into roots, which would explain the decrease in cytokinin content following initiation.

The greatest peak in activity, however, was detected at Days 13 - 15, with a gradual increase to this point. This is consistent with the premise that the elongating and newly emerged roots are important sites of cytokinin synthesis (FEATONBY-SMITH AND VAN STADEN, 1981). The compounds showing the greatest activity at this point co-chromatographed with zeatin and zeatin riboside.

Investigations have been conducted to determine at what stage adventitious root primordia start to contribute to the cytokinin pool of the plant. Using root formation in leaf cuttings of *Phaseolus vulgaris*, it was found that the cytokinin content of the leaf increased throughout the experiment, especially after the sixth day (FEATONBY-SMITH AND VAN STADEN, 1981). This was the point at which some adventitious roots had penetrated the epidermis of the petiole. The newly formed roots were thus concluded to produce cytokinins which were then transported to the leaf lamina where they were required for growth. This contribution was found to occur at an early stage (after 4 days) with a higher rate of synthesis after 8-10 days (FEATONBY-SMITH AND VAN STADEN, 1981).

The eventual sharp increase in cytokinin levels in the lower halves of the *P.euramericana* cuttings with the formation of wound-roots, was due to the

presence of roots (the main source of cytokinins). This was accompanied by a corresponding slight increase in cytokinin levels in the upper halves of cuttings that could be due to the upward transport of cytokinins from the roots. In contrast, the corresponding increase in cytokinin levels in *P. tremula* cuttings, which did not root, is relatively small. The initial decrease in cytokinin activity in both upper and lower halves of cuttings was reversed in the easy-to-root cuttings when roots had formed, and to a lesser extent in the difficult-to-root species when the leaves had expanded. This increase was in the lower halves of the stem cuttings than in the upper halves (OKORO AND GRACE, 1978).

Thus from the above set of results, it would appear that immediately following adventitious root induction there is a substantial increase in the polar compounds exhibiting cytokinin-like activity. Once the roots have emerged from the epidermis and have begun to elongate, there was a significant increase in the non-polar cytokinin-like compounds. This implies differing roles for the different cytokinin types at different developmental stages and starts to explain the differing sensitivities shown by cuttings to the application of cytokinins at various stages of root formation.

It is also evident from these results that this easy-to-root plant undergoes significant changes in both the levels and types of the cytokinins present in the lower portion of the stem cutting during the course of root initiation and development. This lends support to the theory that it is not so much the initial levels and/or cytokinin complement that is important in the ability of the cutting to initiate and develop adventitious roots, but the ability of the stem tissues to change these levels through metabolism (KEFELI, 1978). This thus implies that difficult-to-root species lack this ability.

CHAPTER 5

THE EFFECT OF EXOGENOUSLY APPLIED CYTOKININS AND AUXINS ON ROOT FORMATION

5.1 Introduction

Of the plants that need to be severed from the root system before rooting will occur, some also require the application of growth regulators or other chemicals to initiate this process. It is possible that although these chemicals may affect the anatomical events following their application, not all these changes may be associated with the rooting process (LOVELL AND WHITE, 1986).

With respect to the induction of adventitious root primordia in herbaceous plants, the origin of the primordia is in most cases close to the vascular tissues, irrespective of species or material (LOVELL AND WHITE, 1986). There are few physical barriers to root initiation and development in these plants. Where incomplete rings of fibre bands occur (e.g. *Phaseolus vulgaris*), roots originate in the sectors where the fibre bands are absent. In *Phaseolus* hypocotyls, roots develop in four distinct long rows parallel to, and in between the four pairs of vascular bundles. The application of auxin does not alter the site of origin (LOVELL AND WHITE, 1986). It is also common for four rows of roots to be produced in hypocotyl cuttings of *P. aureus* - they are associated with cotyledonary and primary leaf traces. These roots usually develop near to the cut base but occasionally arise up to 20-30 mm above it.

The mung bean assay was developed by HESS (1961c) for the detection of substances, other than IAA that stimulate root initiation. The hypocotyls of mung bean cuttings differentiate to form adventitious roots when placed in

a suitable environment. Specialised cells of phloem parenchyma alter from the normal non-dividing stage to become meristematic (CHANDRA, GREGORY AND WORLEY, 1971).

Cytokinins were applied in a range of concentrations to cuttings. *Impatiens* cuttings were used to investigate the effect of application of cytokinins on rooting. With this system root length and lateral root initiation can be studied in addition to adventitious root production. The effect of the application of auxins was also investigated to serve as a comparison. The mung bean assay was used as a further experimental system in order to provide a comparison to published research.

5.2 Materials and methods

Impatiens cuttings, standardized with respect to stem diameter and length, were selected for use. These were subjected to an 8-hour pulse in the test hormone solution. The cuttings were then rinsed briefly in distilled water and placed in tubes of distilled water in a Conviron (temperature 25°C, light intensity 32.1 μmol m^{-2} s^{-1}) for 10 days. The length and number of roots produced, as well as the number and average length of lateral roots were recorded. The average values for these measurements were entered in Figure 5.1 and Figure 5.2.

The experiment was repeated using the mung bean bioassay. The method followed is described in Chapter 2. These results are presented in Figure 5.3 and Figure 5.4.

5.3 Results

The treatment of *Impatiens* cuttings with auxin stimulated the number of roots produced (Fig 5.1 A-C).

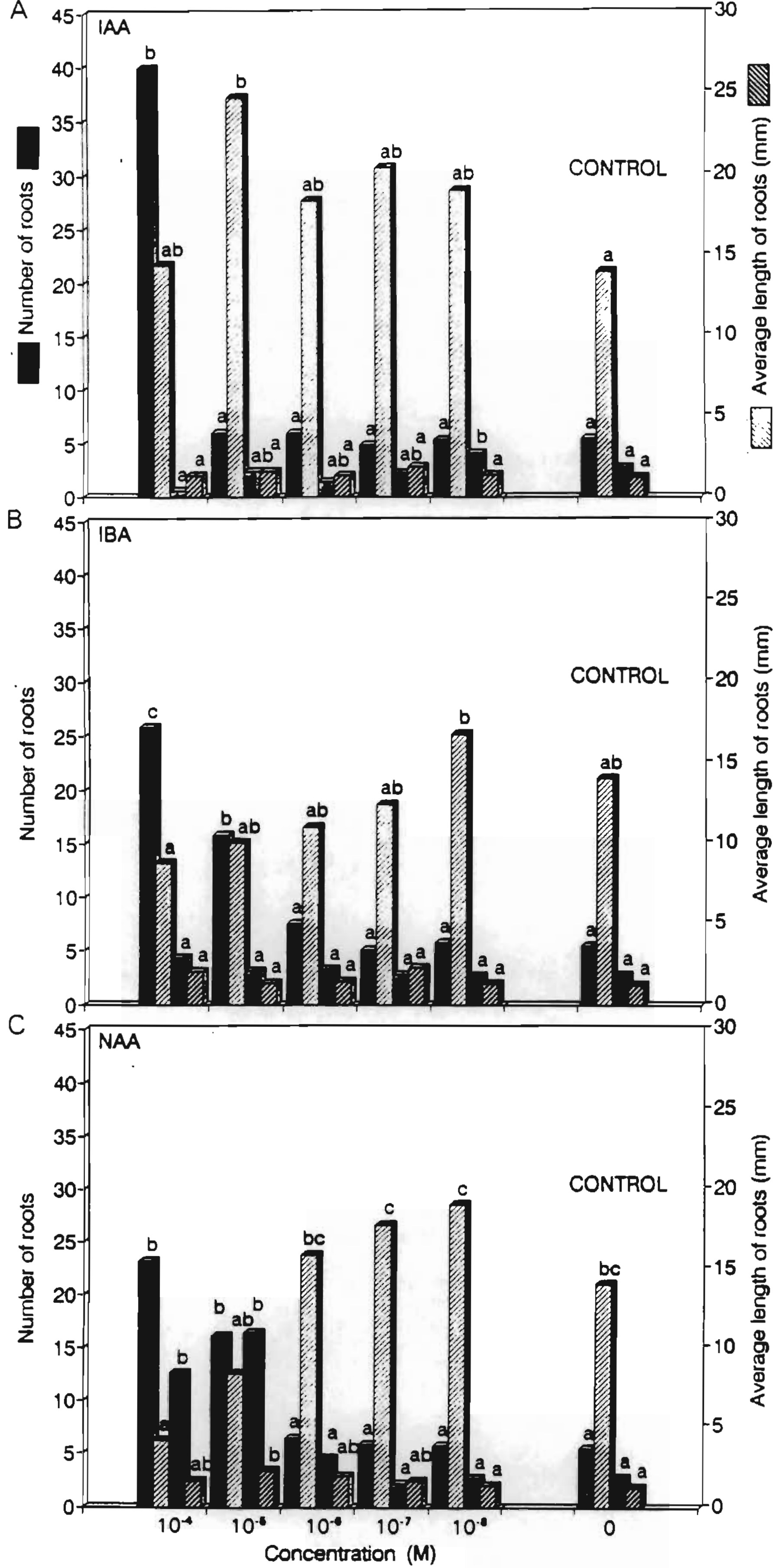


Figure 5.1 The effect of various auxins on root initiation (▬); root length (▭); number of lateral roots produced (▬) and average length of lateral roots (▨) in *Impatiens* stem cuttings. (A) IAA; (B) IBA; (C) NAA. Bars bearing different letters are significantly different, $P \leq 0.05$.

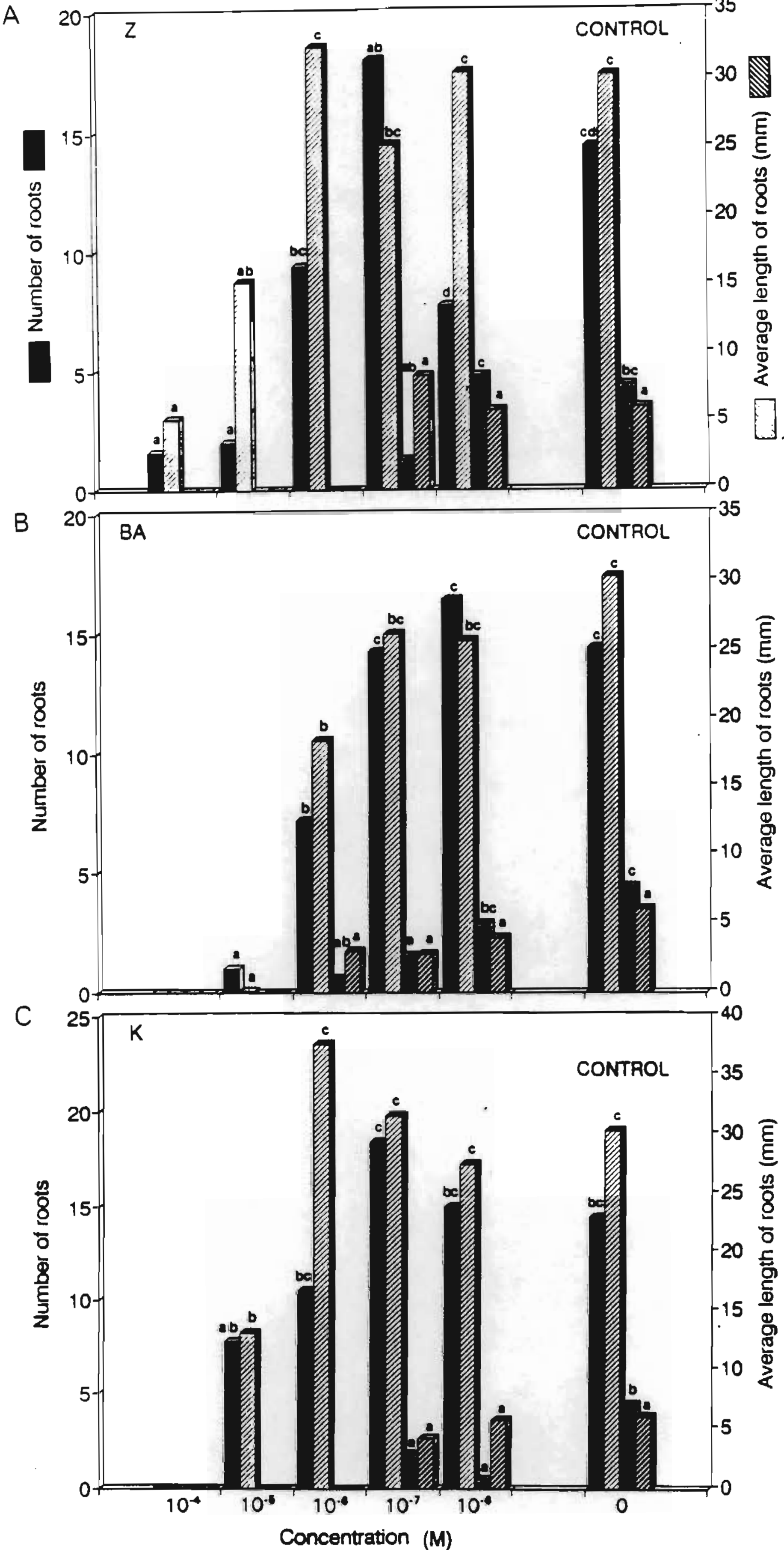


Figure 5.2 The effect of various cytokinins on root initiation (■); root length (▨); number of lateral roots produced (■) and average length of lateral roots (▨) in *Impatiens* stem cuttings. (A) Zeatin; (B) BA; (C) Kinetin. Bars bearing different letters are significantly different, $P \leq 0.05$.

IAA had no significant effect on root length or lateral root emergence, but at high concentrations (10^{-4}M) did significantly stimulate root initiation (Fig 5.1 A). IBA had no significant effect on root initiation and growth within the concentration range tested (Fig. 5.1 B). NAA was inhibitory to root formation and elongation at high concentrations (10^{-4} and 10^{-5} M), while at lower concentrations there was an increase in the number of roots produced, with a corresponding decrease in root length (Fig 5.1 C).

The application of high concentrations of cytokinins inhibits root formation (Figure 5.2 A-C). Zeatin at 10^{-7} M did have a stimulative effect on root number, but this was not accompanied by any increase in root length (Fig. 5.2 A). The application of BA in high concentrations (10^{-4} and 10^{-5} M) proved to completely inhibit root initiation (Fig. 5.2 B). BA at lower concentrations had little effect on root initiation and growth. While kinetin was less inhibitory than BA, application of this cytokinin also inhibited root development at concentrations above 10^{-6} M (Fig. 5.2 C).

The results obtained for the mung bean rooting assay showed more clearly the effect of the different hormone dilution series on rooting. A wider range of cytokinin concentrations was used in this experiment.

IAA had a significant stimulative effect on the number of roots produced for the 10^{-4} M treatment, and on the length of roots at 10^{-5} M. IAA did not significantly alter the root development at concentrations below 10^{-6} M (Fig. 5.3 A). Similarly, IBA had a significant stimulative effect on the number of roots produced at high concentrations (Fig. 5.3 B). There was a trend of increasing root length with decreasing IBA concentration. Application of high concentrations of NAA stimulated both the number of roots formed and the number of lateral roots produced (Fig. 5.3 C). This was accompanied by a decrease in the average root length. There was a corresponding increase in average root length with the decrease in number of roots produced (Fig.5.4 C).

Figure 5.4 A-C illustrates the effect of cytokinins on root development in mung bean cuttings. While Figure 5.4 A shows a trend of increased root number and length with increasing zeatin concentration, with a peak at 10^{-8} M, this was not statistically significant. Similarly, concentrations of BA within the range 10^{-8} - 10^{-10} M increased the length of lateral roots, this too did not prove to be significant. Kinetin showed no significant effect on the number of roots produced but at 10^{-12} M did significantly increase the average length of the roots (Fig. 5.4 C).

5.4 Discussion

While criticism has been levelled at the validity of testing rooting factors in the mung bean rooting bioassay (WILSON AND VAN STADEN, 1991; WILSON, DICKS AND VAN STADEN, 1994), it provides a rapid indication of the effect of these substances on the rooting of cuttings and does establish a means of standardising results by comparison with other published work.

With respect to treatment of cuttings with exogenously applied hormones, it was found that of the auxins, IBA was particularly effective in root induction, closely followed by the naturally occurring IAA. This latter compound is especially sensitive to light, and enzymatic oxidation to which synthetic compounds, e.g. ~~IAA~~ IBA, are not subject (DODDS AND ROBERTS, 1985).

The results from this series of experiments showed that when applied in similar concentrations, cytokinins have a distinct inhibitory effect, as opposed to auxins. At lower concentrations cytokinins have no visible effect, as with auxins. This is in accordance with other work showing weak reactivity of roots to applied zeatin (BOURQUIN AND PILET, 1990).

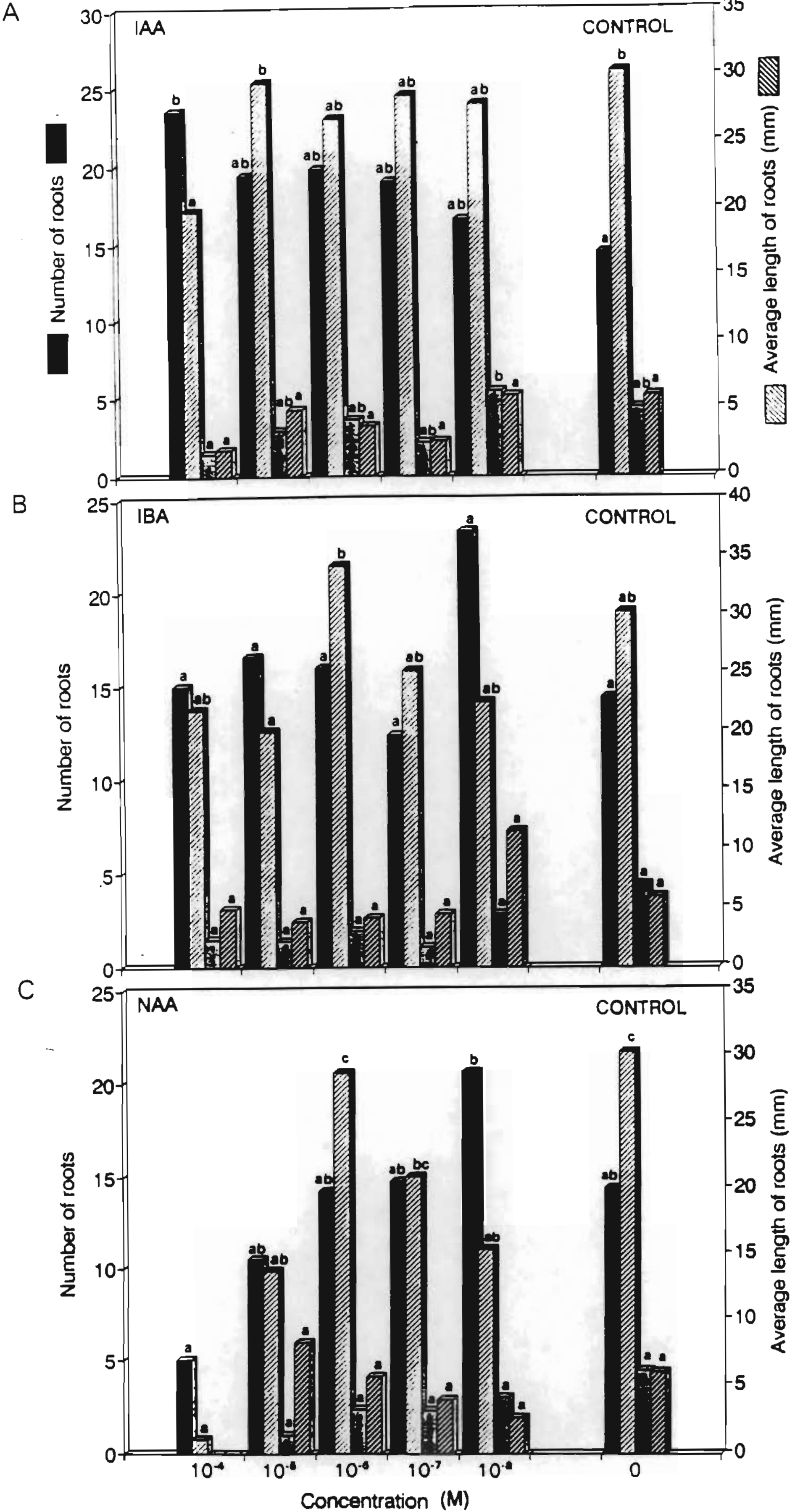


Figure 5.3 The effect of various auxins on root initiation (▬); root length (▭); number of lateral roots produced (▬) and average length of lateral roots (▨) in *Vigna radiata* hypocotyl cuttings. (A) IAA; (B) IBA; (C) NAA. Bars bearing different letters are significantly different, $P \leq 0.05$.

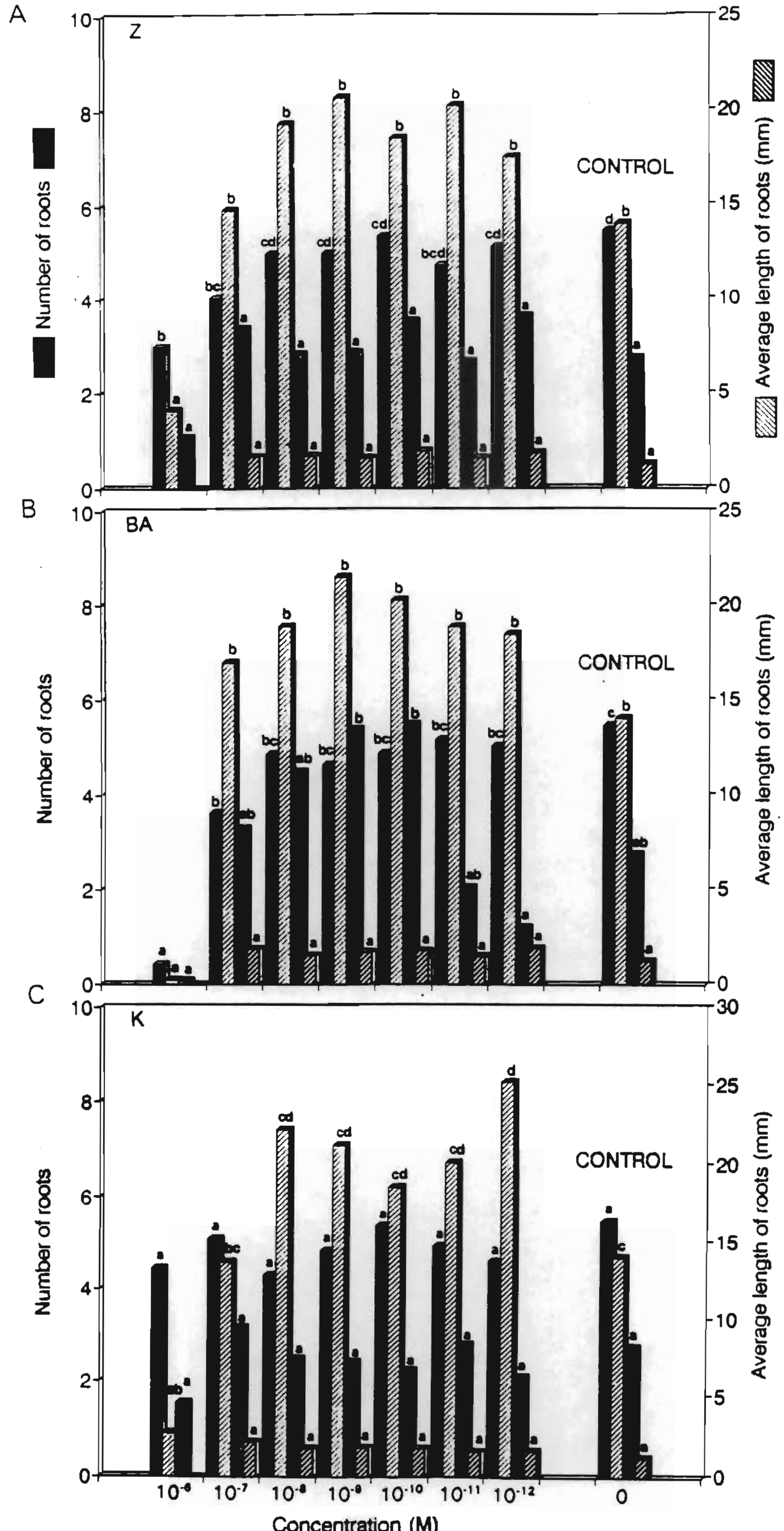


Figure 5.4 The effect of various cytokinins on root initiation (▬); root length (▭); number of lateral roots produced (▬) and average length of lateral roots (▨) in *Vigna radiata* hypocotyl cuttings. (A) Zeatin; (B) BA; (C) Kinetin. Bars bearing different letters are significantly different, $P \leq 0.05$.

The stimulation of rooting by low levels of BA and zeatin, and inhibition by higher quantities, is in accordance with the effect of cytokinins in differentiating cells in culture (FABIJAN, TAYLOR AND REID, 1981). This effect was observed in both experiments (Fig. 5.2 A-C and Fig. 5.4 A-C). A small amount of cytokinin might be a prerequisite for root initiation. In the intact plant, adventitious root initiation is normally blocked by gibberellins and cytokinins from the roots, and the fact that the roots act as a sink for auxin and nutrients is essential in the rooting process. Removal or damage of the roots thus automatically leads to adventitious root formation (FABIJAN, TAYLOR AND REID, 1981).

It was found that, at the higher concentrations of auxin, particularly IBA, root elongation was inhibited (Fig. 5.1 A-C and Fig. 5.3 A-C). Auxin (above 10^{-8} M) is known to inhibit the elongation of roots (THIMANN, 1977). The range of auxin concentrations promoting elongation is very small (THIMANN, 1977) and lies at approximately 10^{-4} M (Fig. 5.1).

Both IAA and IBA have been detected in the root regenerative tissue of untreated (control) *Phaseolus vulgaris* hypocotyl cuttings. There is a distinct increase in the IAA and IBA level in the regenerative tissue after 24 hours of regeneration (BRUNNER, 1978).

The effect of auxin

In both woody and non-woody cuttings a constant supply of auxin is needed throughout the initiation phase of root formation. High concentrations of auxins, even when applied for periods as short as 30 minutes must therefore ensure an adequate dosage to initiate cell division and control the organisation of primordia. Exogenously applied auxin does not, however, persist at high concentrations in the region of regeneration throughout the entire period required for the formation of root primordia. In addition, excessively high concentrations of applied auxin inhibit the growth of the

primordia, and even the initiation itself. Observations suggest that the second phase of auxin action may be associated with lower levels of auxin than is the first phase (JARVIS, 1986).

The existence of an initiation phase in the formation of a root primordium was demonstrated in mung bean cuttings by CHANDRA, GREGORY AND WORLEY (1971). An investigation into the auxin-induced differentiation of lateral roots in primary roots of lettuce seedlings revealed that this is a two-stage process with high auxin levels being required for less than 24 hours to induce cell division in the pericycle (MACISAAC, SAWHNEY AND POHORECKY, 1989). The continued division resulting in the formation of the lateral root primordia was auxin independent (MACISAAC, SAWHNEY AND POHORECKY, 1989).

Once roots emerge and begin to elongate there is a negative reaction to auxin. Auxin inhibits elongation of roots at concentrations above 10^{-8} M (THIMANN, 1977). The zone of auxin concentrations causing promotion is usually very small. This was evident in both experiments (Fig. 5.1 A-C and Fig. 5.3 A-C). Often there is a temporary acceleration in growth, followed by sharp curtailment. Cuttings root best if they are removed from the exogenous source of auxin as soon as root initials have emerged. They are then placed in a non-auxin medium to allow elongation (THIMANN, 1977).

Little is known about the process of lateral or adventitious root formation at the molecular level (PELOSI, LEE, CHANDLER AND HAMILL, 1995). High levels of auxin (10^{-3} M) have been found to be inhibitory of lateral root primordia induction, with levels below 10^{-5} M being stimulative. The development of these primordia into lateral roots is inhibited by continuous incubation in a medium containing auxin. Exposure to these low levels of auxin for a period of 18 - 20 hours, followed by transferral to an auxin free medium, has been found to be optimal (PELOSI, LEE, CHANDLER AND HAMILL, 1995; MACISAAC, SAWHNEY AND POHORECKY, 1989). It was further reported that a high auxin:cytokinin ratio is required for lateral root

primordia induction, with high levels of cytokinins being inhibitory to lateral root formation (WIGHTMAN, SCHNEIDER AND THIMANN, 1980; MACISAAC, SAWHNEY AND POHORECKY, 1989; PELOSI, LEE, CHANDLER AND HAMILL, 1995).

The root promoting effects of auxins decline when their application is delayed. It is proposed that the promotive effects of auxin on the induction of root primordia are opposed by the inhibitory effects of auxin-induced ethylene (feedback). Ethylene inhibits formation of adventitious root primordia in cuttings, but it promotes emergence of roots in stems with preformed primordia. Adventitious rooting is promoted when rates of ethylene production are low relative to auxin concentration (MULLINS, 1972).

Investigation into the effect of ethylene may be complicated by the existence of periods of differing sensitivity to the gas. It is also possible, that even at an early stage, the elongating root primordia start to act like the original root system and exert an inhibitory influence on further root formation (FABIJAN, TAYLOR AND REID, 1981).

Auxin and ethylene are antagonists in root initiation in mung bean cuttings. Auxin promotes rooting but rooting is opposed by the ethylene generated as a consequence of auxin application. In this feedback system, formation of root primordia is promoted when production of ethylene is low relative to concentration of auxin (IBA). With potent inducers of ethylene (IAA) the promotive effects of auxin on rooting are outweighed by inhibitory effects of ethylene (MULLINS, 1972).

For root promotion IAA must be supplied at high concentrations but low volume so that initially sufficient hormone is applied to elicit a rooting response. The internal concentration of IAA is able to subsequently decline rapidly to minimise ethylene production. A compromise must be reached between the requirement for induction of root primordia, the rate of IAA

degradation, and IAA induced ethylene production. (MULLINS, 1972)

A comparison of the ethylene-inducing properties of different auxins could account for their differing effectiveness in the induction of rooting. Research shows that IAA alone has little effect on the rooting of mung bean cuttings. IBA is a potent stimulator of adventitious rooting (MULLINS, 1972). The results presented in Figures 5.1 A-C and 5.3 A-C are in agreement with this, and further indicate the differences in effectiveness between NAA, and IAA and IBA in this regard. Superiority of IBA in rooting has been attributed to its greater resistance to degradation, but IAA has a much greater effect on ethylene production than IBA (MULLINS, 1972).

The effect of cytokinin application

Studies have shown that cytokinins at moderately high levels, can act as potent inhibitors of adventitious rooting. The normal export of cytokinins from roots through the hypocotyl is thought to inhibit primordia initiation prior to excision. Any reduction in the quantity of these hormones - as would occur subsequent to root tip removal - aids in root primordia formation (FABIJAN, TAYLOR AND REID, 1981). In cuttings that have started to produce roots there appears to be a direct inhibitory effect of the mature primordia on the position of the future sites of lateral root initiation.

The early events leading to root initiation occur within a few hours of root excision. The examination of hypocotyls treated with BA at any stage during the first 12 hours following excision showed a lack of any of the cell divisions typical of the earliest stage of root formation (FABIJAN, TAYLOR AND REID, 1981). Preliminary events leading to primordia initiation are most sensitive to the inhibitory action of both cytokinins and gibberellins. The lack of effectiveness of BA when supplied after 30 hours of commencing the experiment, shows that after this time, few new root primordia are initiated (FABIJAN, TAYLOR AND REID, 1981).

It has been suggested that zeatin regulates root elongation by acting on IAA and/or ABA levels (BOURQUIN AND PILET, 1990). In wheat roots, zeatin is the most significant growth inhibitor - a negative correlation exists in maize roots between growth and IAA and ABA levels in the elongation zone. Cytokinin-inhibited conjugation and biosynthesis of IAA and biosynthesis of ABA. Generally cytokinins are reported to powerfully inhibit the formation of lateral roots. Longitudinal wounding may increase root number (THIMANN, 1977).

Root formation in cuttings involves a sequence of phenomena with differing requirements for growth substances (MULLINS, 1972). In mung beans, the effects of kinetin on root primordia formation can be related to the time of application and are relative to concentration of auxin supplied at excision (MULLINS, 1972). Kinetin inhibits formation of primordia when supplied to newly made cuttings but later applications had progressively less effect. Cuttings treated with IBA were less susceptible to inhibition by kinetin at all times of application after 24 hours, suggesting that the auxin:cytokinin ratio is important in rooting of cuttings and in the formation of roots in undifferentiated (callus) tissue (MULLINS, 1972) as indicated by previous research.

CHAPTER 6

THE EFFECT OF CENTRIFUGATION ON THE ROOTING OF CUTTINGS

6.1 Introduction

Centrifugation enhanced root formation in cuttings of a number of woody plants (VAN STADEN, 1978). Root formation in willow and poplar cuttings was enhanced by basipetal centrifugal forces (KAWASE, 1964). This led to the conclusion that the endogenous basipetal transport of rooting substance(s) is enhanced by this treatment. Substances found in the aqueous fraction of the extraction were active in promoting rooting (KAWASE, 1964, 1970). Although auxin has been detected in the diffusate of *Citrus* cuttings (GOLDSCHMIDT AND MONSELISE, 1968), the improvement in the rooting ability was not as a result of increased auxin levels at the base of the cutting (KAWASE, 1964).

Leaves are thought to be capable of supplying the growth substances necessary for the initiation of cambial activity and rooting. Cell division inducing compounds, in association with other hormones, may be responsible for cambial activity (VAN STADEN AND BROWN, 1977), which would include the initiation of adventitious roots. Centrifugation of cuttings leads to the redistribution of cytokinins in the stem. The extent to which cytokinin-like substances are lost from the cutting (when centrifuged in water), is thought to depend on the localization of these compounds in the stem tissue (VAN STADEN, 1978).

Impatiens and *Eucalyptus* stem cuttings were centrifuged before being divided into upper and lower halves. These were analysed to establish their cytokinin

complement. The water used in the centrifugation process were also analysed to determine whether cytokinins were present. Cytokinins are present mostly in the xylem, and would therefore be more easily lost from the vessels than when they are transported in the phloem. It is thus proposed that centrifugation facilitates the removal of cytokinins from the cutting, resulting in reduced cytokinin levels, and a hormone balance that is favourable for rooting.

6.2 Materials and methods

Impatiens, *Eucalyptus grandis*, and *E.macarthuri* cuttings were used in this experiment. No experiments could be conducted with mung beans as the material was too soft. The cuttings were cut to a length of 8 cm and all except the four apical leaves were removed.

Two *Impatiens* cuttings were placed in each centrifuge tube with 4 mℓ distilled water. The cuttings were centrifuged at 1000 rpm (110g) for 10 minutes. This was repeated at 2000 rpm (450g) for 10 minutes with fresh cuttings. This process was then repeated with cuttings placed in dry centrifuge tubes.

The *Eucalyptus* cuttings were subjected to a centrifugal force of 2000 rpm (450g) for 10 minutes. This was repeated for cuttings placed in dry tubes and with 4 mℓ distilled water added.

After centrifugation the stem cuttings were cut in half and each half extracted for cytokinins as before (Chapter 2). Paper chromatography was used to separate the cytokinins, and the ten R_f fractions were assayed using the soybean callus bioassay (Chapter 2). This was to determine whether there was a difference in the cytokinin levels of the two halves as a result of the centrifugal force.

The water added to the centrifuge tubes (referred to as a diffusate) was filtered through Whatman No. 1 filter paper and taken to dryness in front of a fan overnight. This was then resuspended and applied to a Dowex 50W-X8 column as detailed in Chapter 2. The ammonia fraction was resuspended in 80% ethanol and separated using paper chromatography (Chapter 2) and each of the 10 R_f strips was assayed using the soybean callus bioassay.

Centrifuged cuttings were also placed in vermiculite and allowed to root. This was to determine whether centrifugation affects the rooting capacity of the cutting.

6.3 Results

Centrifugation at 1000 rpm (110g) of *Impatiens* cuttings in the absence of water significantly increased the number of roots formed by the cuttings, relative to centrifugation in the presence of water, but not relative to the control (Fig. 6.1). Similarly, at the higher speed of 2000g (450g), the average length of the roots was significantly increased. The addition of distilled water to the centrifuge tubes generally decreased both root number and length, but this was not significant at $P \leq 0.05$ (Fig. 6.1).

In general, centrifugation did not significantly improve the rooting ability of the hardwood cuttings, with roots forming over a period greater than 10 weeks.

The results of the soybean callus bioassays indicate that centrifugation led to a redistribution of cytokinins in the stem cuttings. Cytokinins co-chromatographing with zeatin and zeatin riboside were present in all the extracts (Fig. 6.2 - Fig. 6.6). These compounds exhibiting cytokinin-like activity are released into the water during "wet" centrifugation.

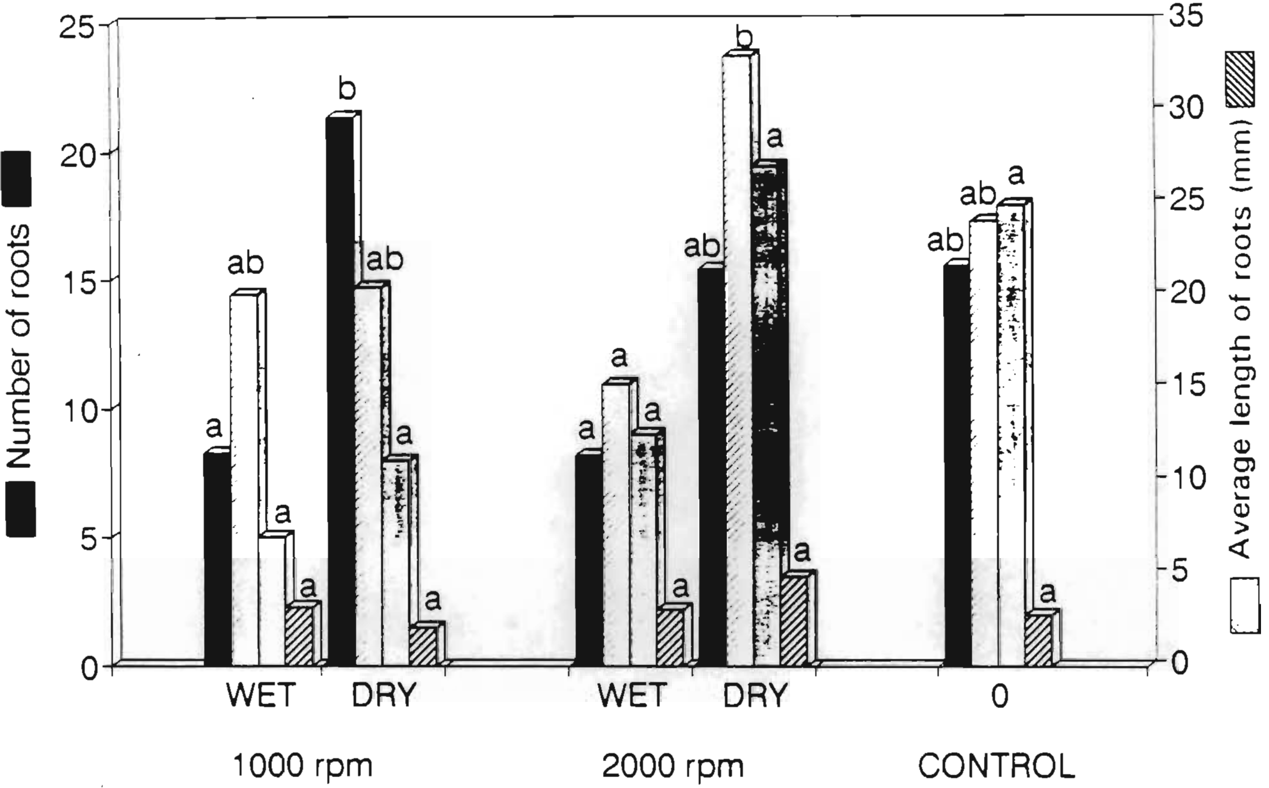


Figure 6.1 The effect of centrifugation on root formation in *Impatiens* stem cuttings. Centrifugal speeds were 1000 rpm (110 g) and 2000 rpm (450 g) for 10 minutes. Number of roots ( ); Average length of roots ( ); Average number of lateral roots ( ); Average length of lateral roots ( ) were recorded. Bars bearing different letters are significantly different, $P \leq 0.05$.

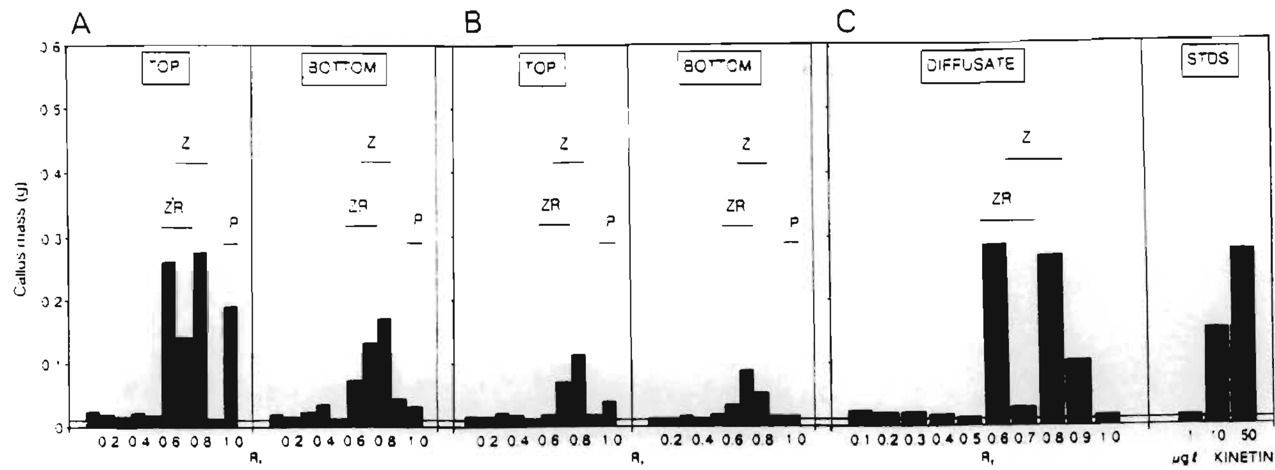


Figure 6.2 The effect of centrifugation at 1000 rpm (110 g) for 10 minutes on the cytokinin complement of *Impatiens* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) Dry centrifugation; (B) Wet centrifugation; (C) Diffusate.

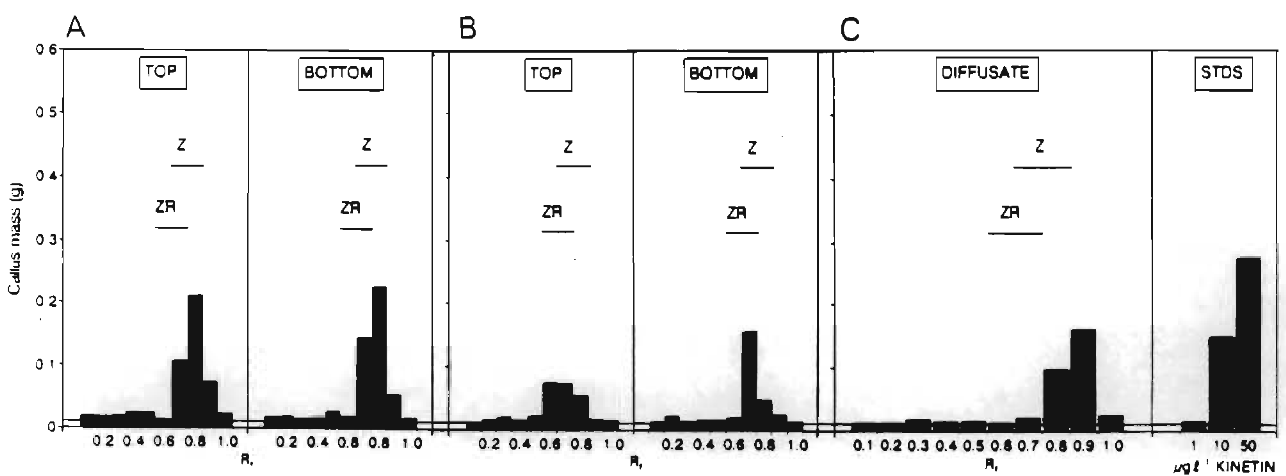


Figure 6.3 The effect of centrifugation at 2000 rpm (450 g) for 10 minutes on the cytokinin complement of *Impatiens* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) Dry centrifugation; (B) Wet centrifugation ; (C) Diffusate.

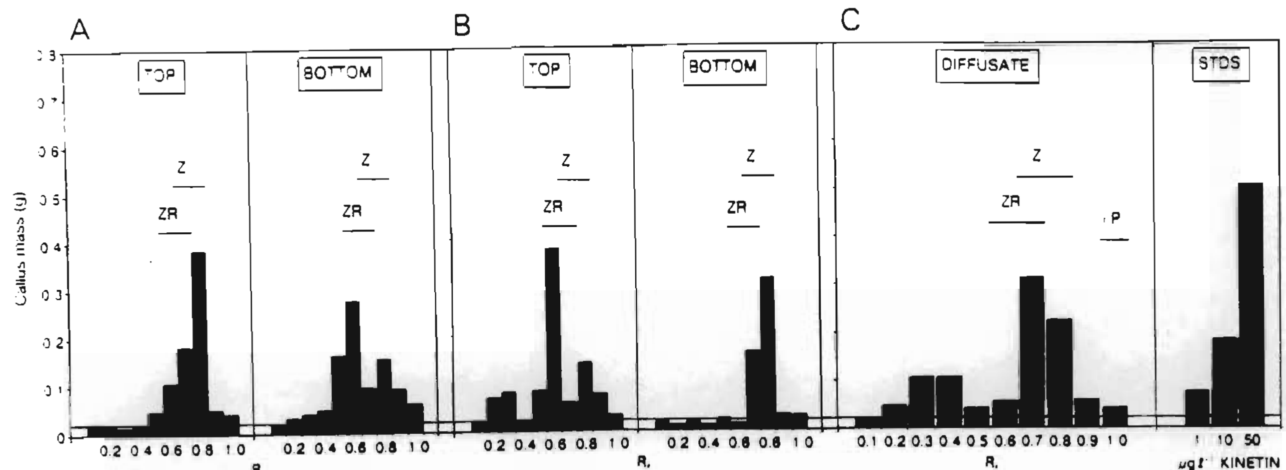


Figure 6.4 The effect of centrifugation at 2000 rpm (450 g) for 10 minutes on the cytokinin complement of *Eucalyptus grandis* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) Dry centrifugation ; (B) Wet centrifugation ; (C) Diffusate.

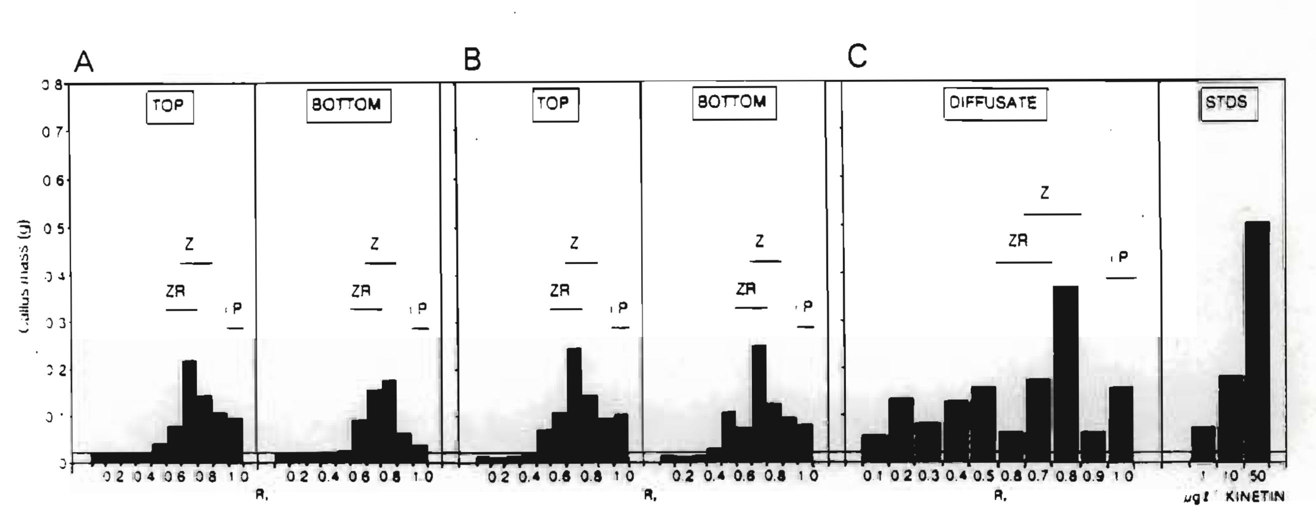


Figure 6.5 The effect of centrifugation at 2000 rpm (450 g) for 10 minutes on the cytokinin complement of *Eucalyptus macarthuri* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) Dry centrifugation ; (B) Wet centrifugation ; (C) Diffusate.

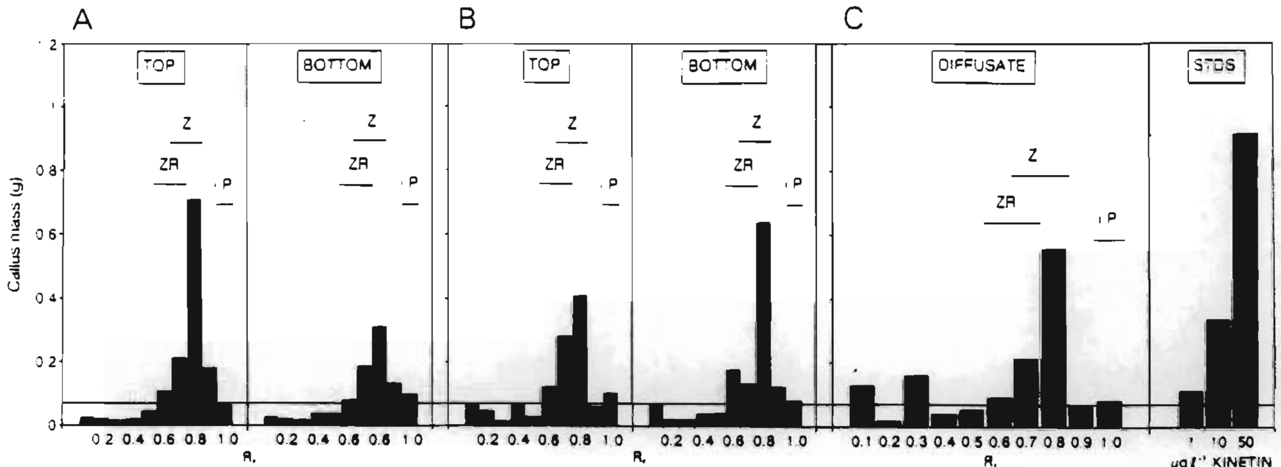


Figure 6.6 The effect of centrifugation at 2000 rpm (450 g) for 10 minutes on the cytokinin complement of *Eucalyptus nitens* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) Dry centrifugation ; (B) Wet centrifugation ; (C) Diffusate.

It is evident that, quantitatively, the cytokinin-like activity was less in the extracts from the *Impatiens* stem material centrifuged with water (Fig. 6.3 B and Fig. 6.4 B) and this corresponded to the activity yielded by the diffusate (Fig. 6.3 C and 6.4 C).

The soybean callus bioassay results from the *Eucalyptus* stem extracts indicated the presence of cytokinin-like compounds in the diffusates (Fig. 6.4 C - Fig. 6.6 C). There was, however, little difference between the levels of cytokinin-like activity detected in the cuttings centrifuged with and without the addition of water (Fig. 6.4 - Fig. 6.6). The cytokinin-like activity for all three *Eucalyptus* species proved similar (Fig.6.4 -Fig. 6.6).

6.4 Discussion

KAWASE (1964) found that root formation was not promoted when willow cuttings were centrifuged basipetally without the addition of water to the centrifuge tubes, or when the basal 2-4 cm of the stem cutting was removed after centrifugation. These findings were countered by those of VAN STADEN (1978), which indicated that dry centrifugation of *Salix babylonica* cuttings increased their ability to form roots, while wet centrifugation resulted in fewer roots. The results presented above (Fig. 6.1) add credibility to the latter findings. The number and length of roots was found to be significantly higher in cuttings centrifuged in the absence of water (Fig. 6.1). It is thus probable that compounds stimulating the initiation and development of roots were released into the diffusate.

The presence of leaves and axillary buds on the cuttings did not modify the effect of centrifugation (KAWASE, 1964). The centrifugal diffusate was found to promote moderate root formation in willow cuttings and strong root formation in mung bean cuttings. It was found to have a strong synergistic effect with IAA in mung bean cuttings (KAWASE, 1964; 1970). This,

together with the evidence of cytokinin-like activity in the diffusates tested above (Fig. 6.2 - 6.6 C), suggest that this root-promoting diffusate contains cytokinins. KAWASE (1964) concluded that the rooting substance(s) in the diffusate was similar to rhizocaline. Basipetal centrifugation accelerated the basipetal transport of this substance(s) and its accumulation in the basal (proximal) ends of the cuttings (KAWASE, 1964).

These results reiterate the importance of cytokinins in the process of root formation. Centrifugation results in the redistribution of both auxins and cytokinins, with an ensuing accumulation of these substances at the base of the cutting. Since the centrifugal force acts on all groups of growth substances, including auxins, cytokinins and gibberellins (KAWASE, 1964; GOLDSCHMIDT AND MONSELISE, 1968; VAN STADEN, 1978), it is probable that the ratios of these compounds remain similar to the original values. In addition, the ability of the tissues to metabolise growth substances such as cytokinins should not alter. This does not, therefore, negate the theory that it is the balance of both stimulators and inhibitors in the cuttings that is responsible for the onset of rooting.

CHAPTER 7

THE EFFECT OF TREATMENT WITH POTASSIUM PERMANGANATE ON THE ROOTING OF CUTTINGS

7.1 Introduction

It is known that potassium permanganate - an oxidising agent - destroys the double bond in zeatin, thus irreversibly destroying its activity. Cytokinin oxidase is the enzyme responsible for the natural catalysis of this cleavage. All compounds with a double bond in the N^6-isoprenoid side chain (including zeatin, zeatin riboside and iso-pentenyladenosine) are susceptible to oxidative cleavage to give adenine, adenosine and adenine nucleotides (McGAW, SCOTT AND HORGAN, 1984; McGAW, 1987). During this reaction molecular oxygen is utilised as the oxidant (HARE AND VAN STADEN, 1994). Oxidising agents such as potassium permanganate, or hydrogen peroxide thus could substitute for oxygen in this reaction, and so potentially alter the active pool of cytokinin-like compounds available at the base of the cutting.

ZIMMERMAN (1930) conducted experiments relating the oxygen content of the water to the ability of a cutting to produce roots. The experiments were run for two weeks and up to 3 cm^3 of hydrogen peroxide was added to the water each week. Both this treatment, and the immersion of the stem cutting in a weak solution of potassium permanganate, improved the rooting capacity of cuttings along the entire length of the stem (although not as effectively as with aerated water).

The following experiment aimed to determine the effect of the oxidising agent potassium permanganate on the rooting ability of stem cuttings. Further, it was ascertained if these treatments affect the cytokinin complement of the stem cutting, and thus whether by changing the levels of active cytokinins in this manner it is possible to improve the rooting capacity of the cutting.

7.2 Materials and methods

The mung bean rooting assay (Chapter 2) was used to test the effect of potassium permanganate on root formation. A dilution series of potassium permanganate solutions was established. Cuttings taken from ten-day-old mung beans were subjected to pulses of 4, 8, 16, and 32 hours in the solutions. After 10 days the number and length of the roots were measured. Subsequent experiments utilized an 8-hour pulse time.

A further experiment was conducted to investigate the effect of leaving the cuttings in the oxidising solution over the entire experimental period. The potassium permanganate solution was thus used as the rooting medium. The cuttings were maintained in a Conviron under the conditions outlined before (Chapter 2).

The first part of the above experiment was repeated using *Impatiens* stem cuttings as the test material. An 8-hour pulse time was used. Two dilutions (0.005 and 0.05%) of potassium permanganate were chosen. *Impatiens* stem cuttings were subjected to an 8-hour pulse in these respective solutions before being divided into upper and lower portions. The extraction and soybean callus bioassay procedures described in Chapter 2 were followed.

7.3 Results

Figure 7.1 illustrates the result of various pulse times on the effect of potassium permanganate on root formation. At both dilutions tested (0.01% and 0.005% wt/vol.), there was no significant difference in the number of roots produced.

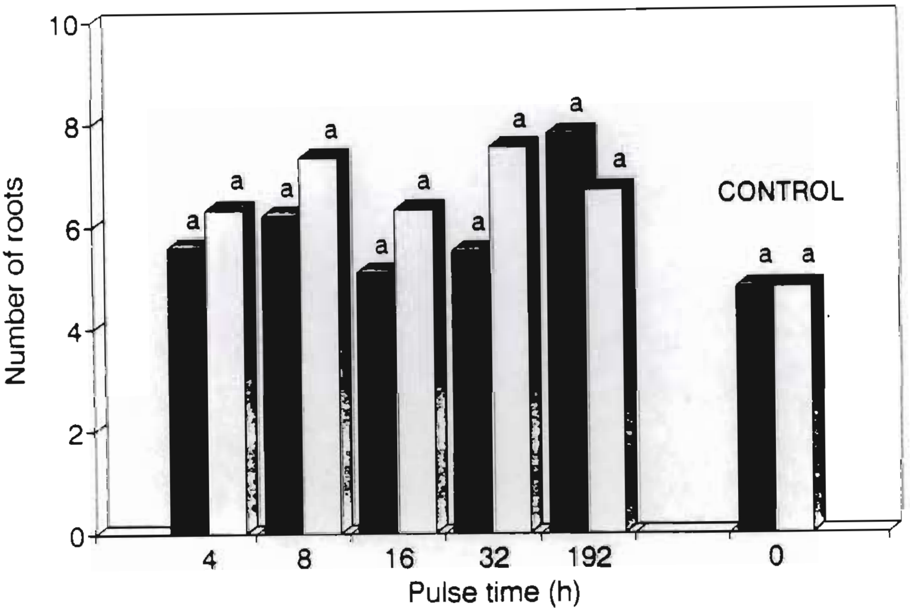


Figure 7.1 The effect of varying the immersion time in potassium permanganate on the number of roots produced by *Vigna radiata* hypocotyl cuttings.

■ 0.01% Potassium permanganate.

□ 0.005% Potassium permanganate.

Bars bearing different letters are significantly different, $P \leq 0.05$.

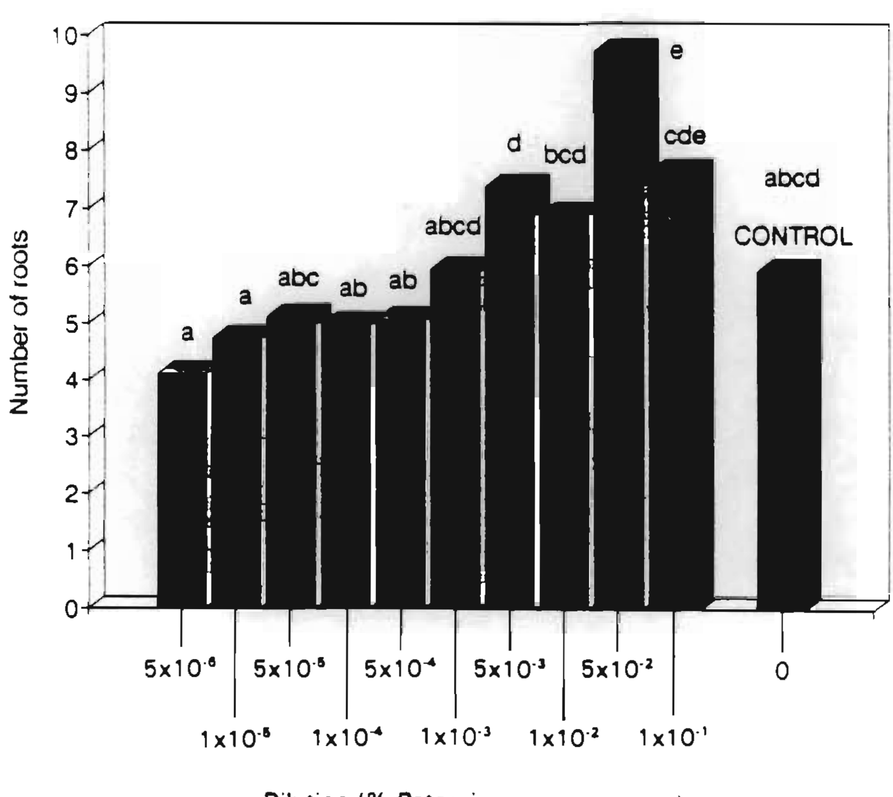


Figure 7.2 The effect of potassium permanganate (8 h pulse) on the number of roots produced by *Vigna radiata* hypocotyl cuttings. Bars bearing different letters are significantly different, $P \leq 0.05$.

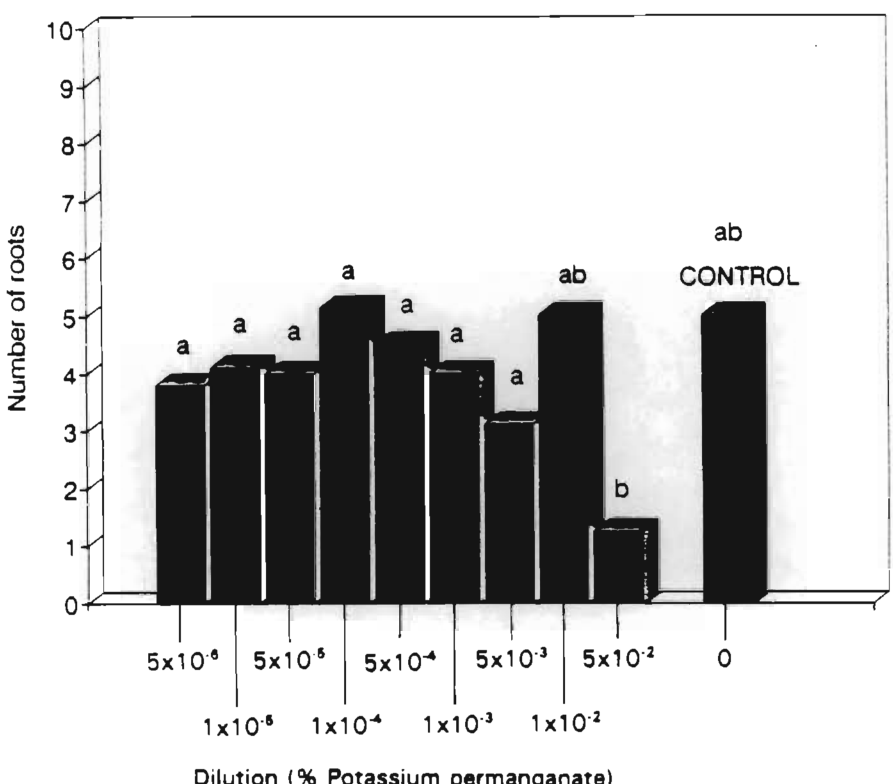


Figure 7.3 The effect of potassium permanganate on the number of roots produced by *Vigna radiata* hypocotyl cuttings. Test dilution of potassium permanganate used as the rooting medium. Bars bearing different letters are significantly different, $P \leq 0.05$.

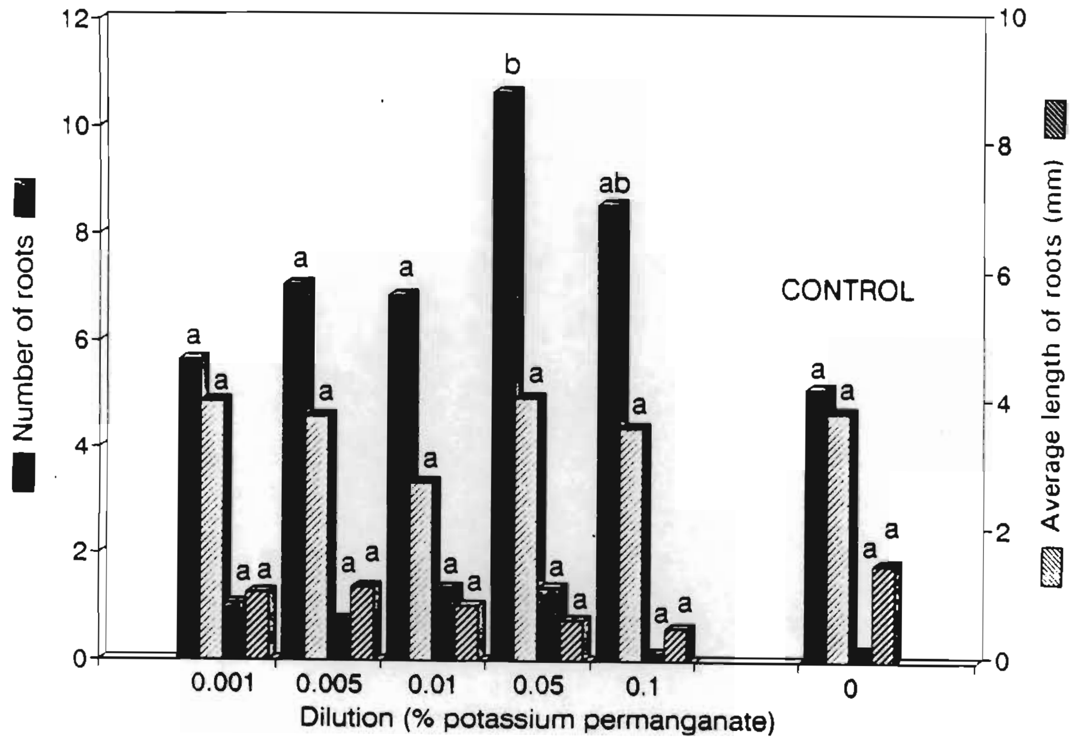


Figure 7.4 The effect of potassium permanganate (8 h pulse) on root formation in *Vigna radiata* cuttings. Number of roots (■); Average length of roots (□); Average number of lateral roots (■); Average length of lateral roots (■) were recorded. Bars bearing different letters are significantly different, $P \leq 0.05$.

Cuttings were then subjected to an 8-hour pulse according to a dilution series. Figure 7.2 shows that the oxidising agent had an increasingly inhibitory effect at dilutions below 0.001%. Within the concentration range tested there was no significant inhibition of rooting. At higher concentrations there was a slight stimulative effect and cuttings treated with 0.05% potassium permanganate produced significantly more roots than the control.

Where the cuttings were placed in the oxidising solution for the entire duration of the experiment, there was an inhibitory effect on root formation, particularly at the highest concentration of 0.5% (Fig. 7.3). At all other concentrations tested there was no significant effect on root formation.

The effect of an 8-hour pulse treatment with potassium permanganate on root number, root length, number of lateral roots and length of lateral roots in mung bean cuttings is presented in Figure 7.4. This treatment had no significant effect on the length of either roots or lateral roots, or on the number of lateral roots produced. The 0.05% treatment significantly increased the number of roots produced by the mung bean cuttings.

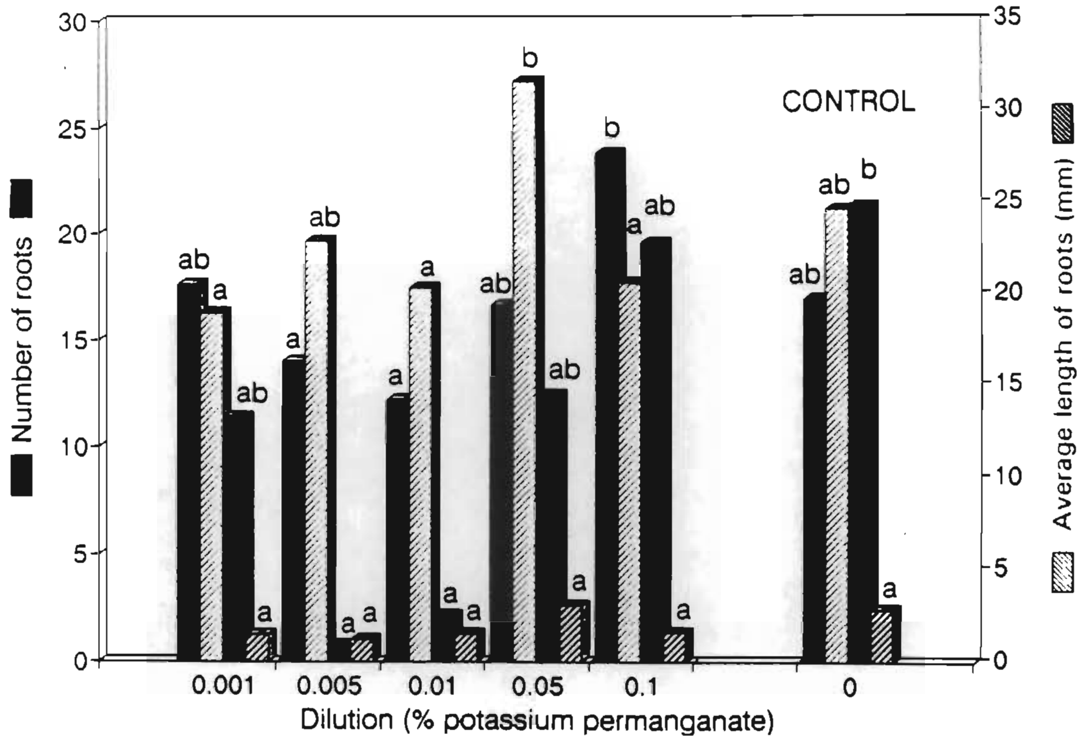
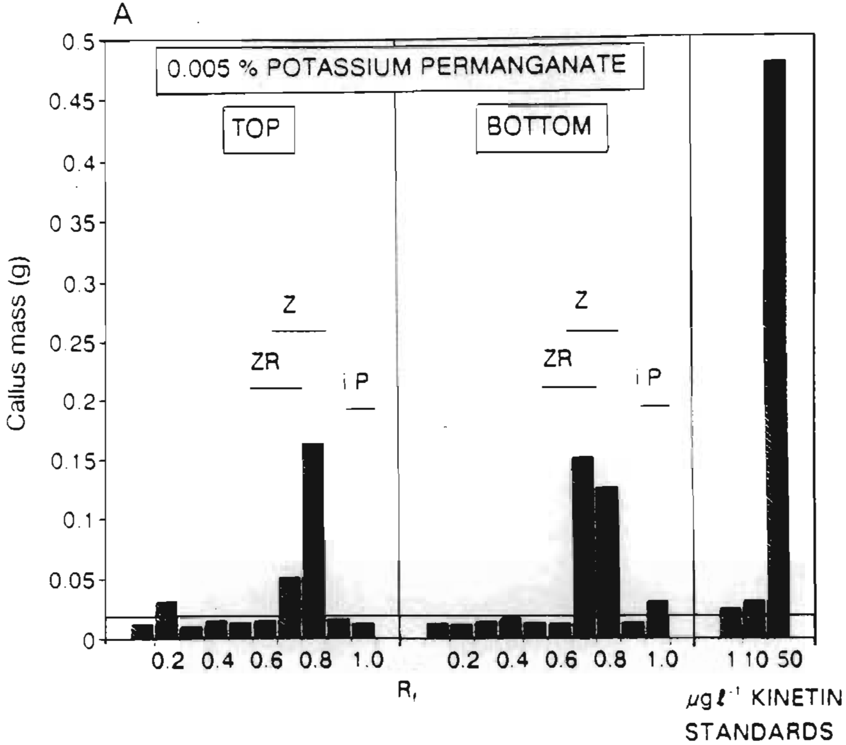


Figure 7.5 The effect of potassium permanganate (8 h pulse) on root formation in *Impatiens* stem cuttings. Number of roots (■); Average length of roots (□); Average number of lateral roots (■); Average length of lateral roots (▨); were recorded. Bars bearing different letters are significantly different, $P \leq 0.05$.



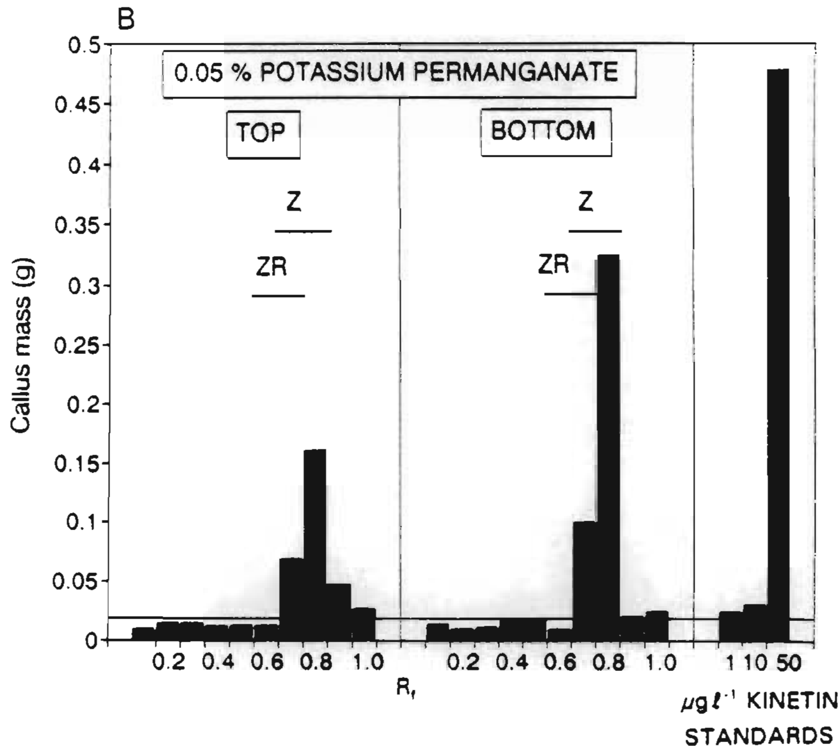


Figure 7.6 The effect of potassium permanganate (8 h pulse) on the cytokinin complement of *Impatiens* stem cuttings. Soybean callus bioassays of extracts from cuttings subjected to (A) 0.005 % Potassium permanganate (B) 0.05 % Potassium permanganate.

The previous experiment was repeated using *Impatiens* stem cuttings (Fig. 7.5). The results showed a similar trend to those in Figure 7.4 but none of these differences proved significant.

The effect of potassium permanganate on the cytokinins in the stem cuttings is presented in Figure 7.6 A&B. The two treatments (0.005% and 0.05% potassium permanganate) yielded qualitatively similar results. Quantitatively higher levels of cytokinin-like activity co-chromatographing with zeatin were present in the cuttings subjected to 0.05% potassium permanganate (Fig. 7.6 A).

7.4 Discussion

Changing the immersion time (4 h; 8 h; 32 h) in potassium permanganate where the test solution was used as the rooting medium, there was a negative effect on the cuttings at high concentrations. This was manifest visibly in the wilting of the cutting, followed by senescence (Fig. 7.3). At the higher concentrations root formation occurred on the upper portion of the hypocotyl, with the lower portion dying. These cuttings did not necessarily senesce, occasionally resulting in a healthy, rooted cutting.

There was a very narrow concentration range in which potassium permanganate treatments promoted rooting (Fig. 7.4). Generally this occurred at 0.05% potassium permanganate. This was thus the concentration chosen to determine the effect of this oxidising agent on the cytokinins present in *Impatiens* stem cuttings. A second dilution (0.005%) was chosen for comparison.

There were no significant differences in the levels or types of cytokinin-like compounds extracted from the stems (Fig. 7.6 A&B). This correlated with the lack of significant differences in the number and length of roots produced by

these cuttings (Fig. 7.5). It is possible that the different cuttings react distinctly to various dilutions of the oxidising agent and that the correct dilution was not used. In addition, these two plant species (*Impatiens* and *Vigna radiata*), have initially different cytokinin complements (Chapter 3).

It would thus appear that the oxidising solution, potassium permanganate, does not, as previously reported (ZIMMERMAN, 1930), stimulate rooting. Comparison with these previous experiments was complicated by the failure of ZIMMERMAN to report the concentrations of oxidising agents (potassium permanganate) used.

CHAPTER 8

THE EFFECT OF CYTOKININ AND AUXIN ON THE GROWTH OF ROOTS IN CULTURE

8.1 Introduction

The culture of excised roots requires the use of a liquid medium. This system has several inherent advantages over the use of nutrients in an agar-solidified matrix. There are no diffusion gradients for nutrients or gases that could lead to altered growth and metabolism. In addition, the agar itself has been found to release contaminants into the culture medium, and metabolites secreted by the explant accumulate in the agar matrix, a problem not encountered in liquid medium (DODDS AND ROBERTS, 1985).

The continuous culture of plant roots can yield important information on the nutritional and hormonal requirements of the root, since the organ has been isolated from the interchange of compounds with other plant organs. Further advantages lie in the elimination of the complicating effects of micro-organisms through the use of sterile techniques. Root clones show a rapid growth rate and are able to multiply rapidly to yield the experimental material required (DODDS AND ROBERTS, 1985).

One possible disadvantage of this type of growth medium is the availability of oxygen and the extent to which the isolated organs require agitation. The importance of these factors is disputed. Early work conducted by STREET (1957, 1969) claimed that oxygen was not a limiting factor in the growth of tomato roots in culture. It was, however, found that a continuous, gentle agitation of tomato root cultures had a significant effect on root elongation, and improved the production and elongation of lateral roots (SAID AND

MURASHIGE, 1979 cited in DODDS AND ROBERTS, 1985). Anaerobic conditions reduce the root dry weight, but not to the extent found with the application of BA and gibberellins (JACKSON AND GOSS, 1978).

The first successful root culture technique was established by WHITE in 1934. Since then several modifications of the media used, have been adopted (DODDS AND ROBERTS, 1985).

Some differences have been observed between cultured excised roots and those produced on an intact plant. Cultured roots gradually lose the ability to produce secondary vascular tissues and fail to show a normal geotropic response. In addition, the biochemical composition of excised roots may differ significantly from those in the intact plant. Despite these differences, DODDS AND ROBERTS (1985) maintain that the results obtained from the use of root cultures in research, are relevant to the physiological activity of the roots of higher plants.

Lateral root formation involves the initiation of several new meristematic areas in the differentiated tissues of the primary root and their subsequent development into mature structures. Cytokinins have been found to have a dual role in lateral root formation, depending on concentration (MACISAAC, SAWHNEY AND POHORECKY, 1989).

Both NAA and kinetin influence the initiation of lateral root primordia in lettuce seedlings. The formation of a functional lateral root is a two-phase process, comprising the initiation of a lateral root primordium and its subsequent emergence from the primary root (MACISAAC, SAWHNEY AND POHORECKY, 1989).

These phases differ in their response to NAA. NAA stimulates the production of lateral root primordia but inhibits the emergence of lateral roots. Cell division is primarily involved in the initiation of lateral roots, while the

emergence involves cell elongation (mainly in the basal zone of the primordium). This difference in the underlying cellular processes of these two phases could be the cause of the differing responses to NAA. Some NAA-induced processes involved in cell division are thought to be sensitive to kinetin, but it is not known what these are. In lettuce roots, kinetin inhibits both the NAA-induced initiation of lateral root primordia, and the subsequent development of lateral root primordia. This latter process is NAA-independent. These factors suggest that NAA and kinetin do not interact directly in the regulation of lateral root initiation (MACISAAC, SAWHNEY AND POHORECKY, 1989).

The aim of the following experiments was to investigate the effect of various auxins and cytokinins on the growth of *in vitro* grown tomato roots. The effect of the cytokinins 6-benzylaminopurine (BA), zeatin (Z), and kinetin (K), was compared to that of the auxins naphthalene acetic acid (NAA), indolebutyric acid (IBA), and indoleacetic acid (IAA). In a second experiment, the effects of zeatin glucoside (ZG), zeatin, zeatin riboside (ZR) and dihydrozeatin (DHZ) were compared. A control was established using the stock medium (no hormones added). The range of concentrations for the auxins, was 10^{-5} to 10^{-13} M, and for the cytokinins, was 10^{8} to 10^{13} M. In addition, the effect of stem extracts from the plants under investigation was tested.

8.2 Materials and methods

In vitro grown tomato roots, maintained in a liquid full strength MILLER's medium, were used as the assay system. The various hormone concentrations were made up with the media and sterilized using an autoclave. The apical 12 mm of lateral roots were transferred to the various test solutions (10 cm^3 in each flask) under sterile conditions on a laminar flow bench. The cultures were agitated on a rotary shaker (120 rpm) to improve

growth (DODDS AND ROBERTS, 1985). Ten replicates were established for each treatment. The cultures were allowed to grow for 10 days. After this period the length of the original root segment (or root axis), and the number and length of any lateral roots were recorded. The experiment was repeated three times and the average values obtained from the data were plotted in Figures 8.1 - 8.5.

In a second experiment, stem material from the various test plants was extracted for cytokinins (divided as before into top and bottom fractions). The crude extracts were separated on paper (Chapter 2), without being applied to Dowex columns, and divided into ten R_f fractions. These were autoclaved in the culture medium (excluding hormones) and removed before the addition of root sections.

8.3 Results

IAA, at 10^{-9} M significantly increased the length of the original root section (axis). Lower concentrations of IAA increased the number of lateral roots produced, but not to a significant extent (Fig. 8.1 A). Figure 8.1 B illustrates the effect of IBA on root development. These results indicate the promotive effect of IBA on axis length at 10^{-7} M and 10^{-11} M. At concentrations lower than 10^{-7} M there was also some increase in lateral root number and length, but this was not statistically significant.

NAA evidently had the greatest effect on the number of lateral roots produced, with the optimal range of application being 10^{-7} to 10^{-9} M (Fig. 8.1 B). In addition, NAA increased the average length of lateral roots. The length of lateral roots was increased optimally at 10^{-11} M (Fig. 8.1 C).

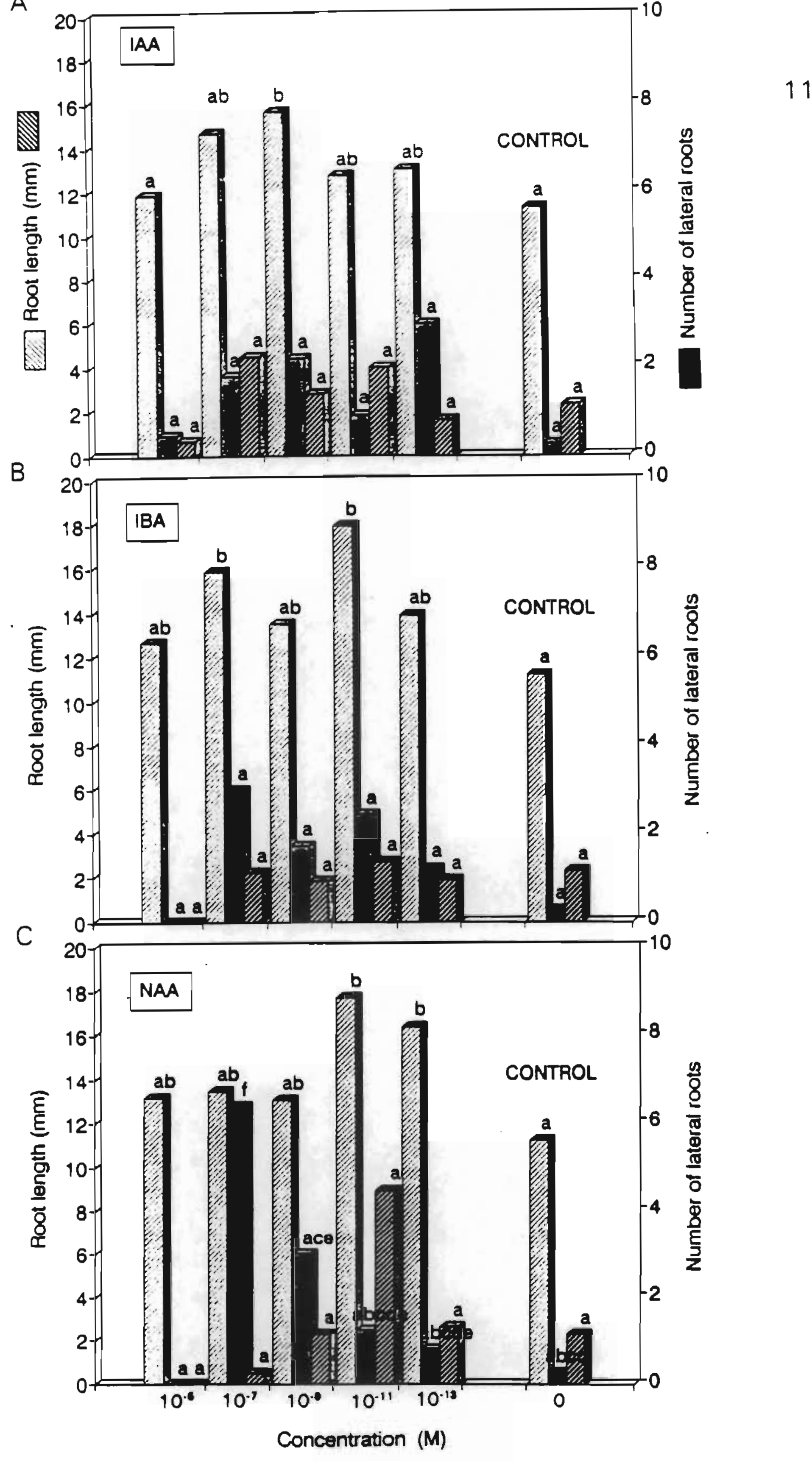


Figure 8.1 The effect of various auxins on development of *Lycopersicon esculentum* roots grown in suspension culture. Root length (▭); number of lateral roots produced (■) and average length of lateral roots (▨) were recorded. (A) IAA; (B) IBA; (C) NAA. Bars bearing different letters are significantly different, $P \leq 0.05$.

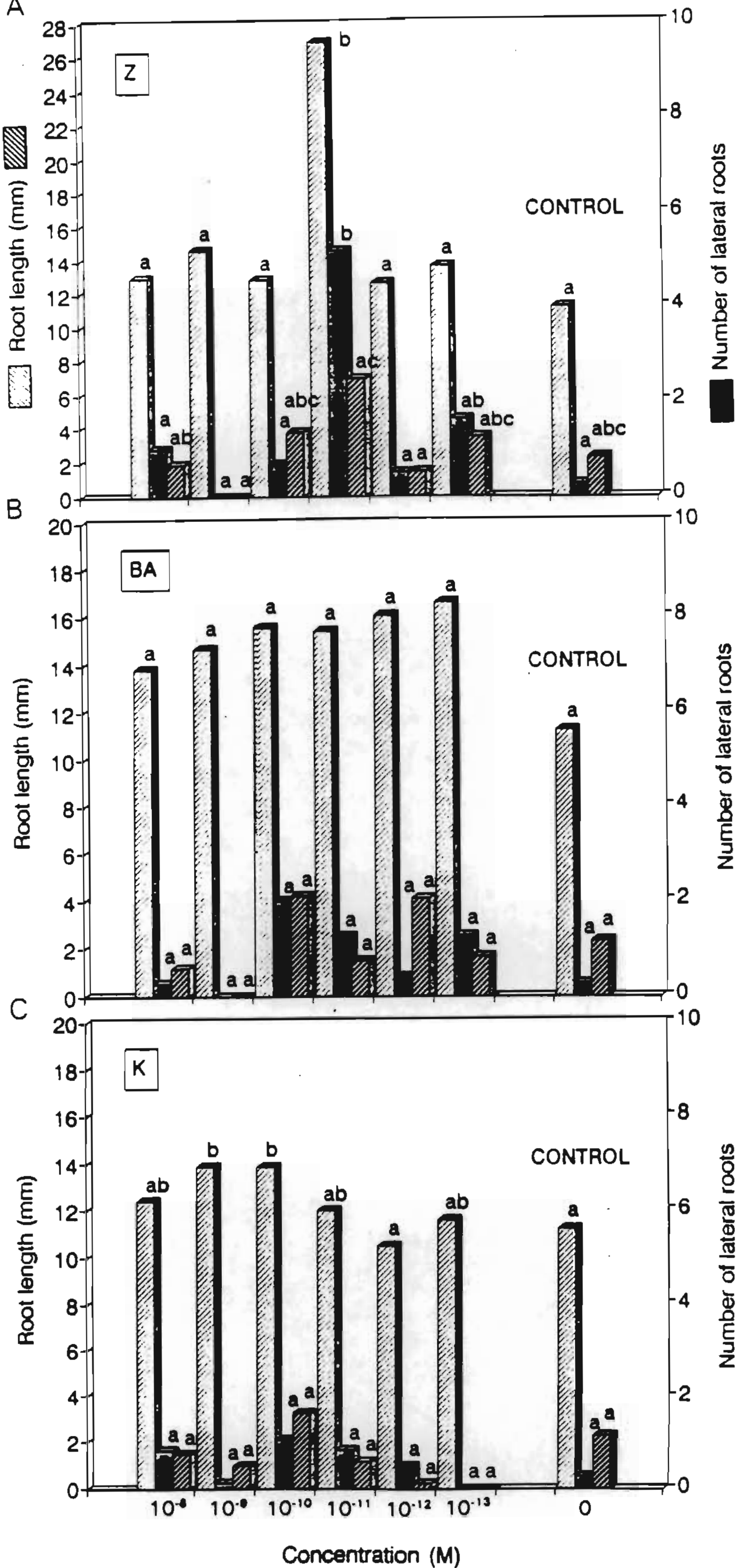


Figure 8.2 The effect of various cytokinins on development of *Lycopersicon esculentum* roots grown in suspension culture. Root length ( ); number of lateral roots produced ( ) and average length of lateral roots ( ) were recorded. (A) Zeatin; (B) BA; (C) Kinetin. Bars bearing different letters are significantly different, $P \leq 0.05$.

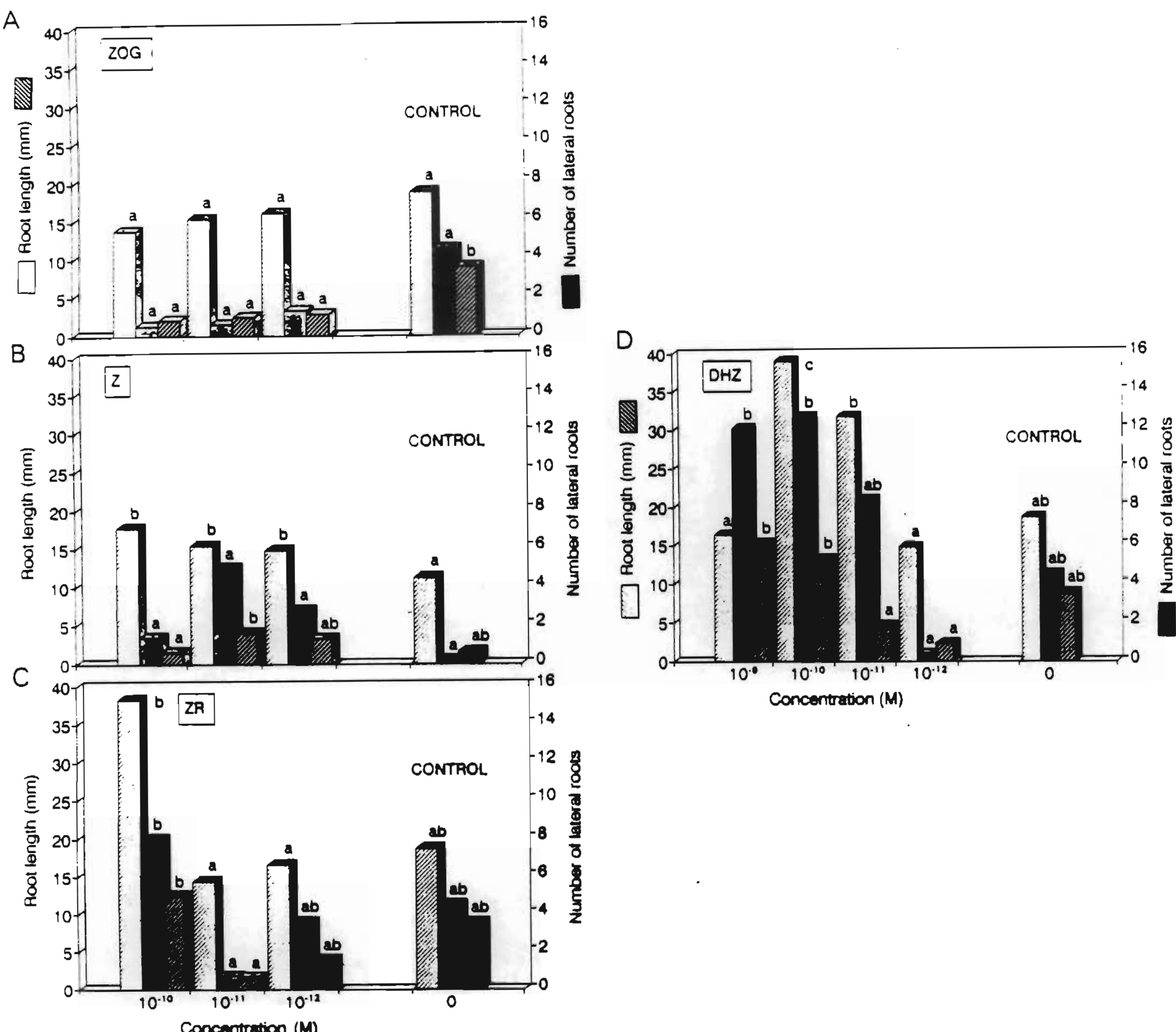


Figure 8.3 The effect of various cytokinins on development of *Lycopersicon esculentum* roots grown in suspension culture. Root length (▭); number of lateral roots produced (■) and average length of lateral roots (▨) were recorded. (A) Zeatin glucoside; (B) Zeatin; (C) Zeatin riboside; (D) Dihydrozeatin. Bars bearing different letters are significantly different, $P \leq 0.05$.

Zeatin stimulated the growth of the axis, and the number and length of lateral roots at low concentrations (10^{-11} M) (Fig. 8.2 A). Low levels of BA in the culture medium allow an increase in the average length but a decrease in the number of lateral roots produced. These were not, however, statistically significant (Fig. 8.2 B). The effect of kinetin (Fig. 8.2 C) was evident at higher concentrations (10^{-9} - 10^{-10} M) and was manifest as an increase in length of the axis and, at 10^{-10} M, of the lateral roots.

In the second experiment, zeatin glucoside had no significant effect on root initiation or growth (Fig. 8.3 A). The concentrations of zeatin tested significantly improved root initiation and growth (Fig. 8.3 B). Although zeatinriboside appeared to increase root length and lateral root initiation, this did not prove to be statistically significant (Fig. 8.3 C). Dihydrozeatin significantly increased the length of the original root segment, but had little effect on lateral root growth. The effect of this cytokinin decreased with decreasing concentration (Fig. 8.3 D).

The various stem extracts had no significant effect on root axis length, and appear to be inhibitory with respect to lateral root formation in culture (Fig. 8.4 and Fig. 8.5). Several R_f fractions from the ***Eucalyptus*** species improved the growth of lateral roots (Fig. 8.5). The fractions yielding activity in this assay showed no correlation with those exhibiting activity in the soybean callus bioassay (Chapter 3).

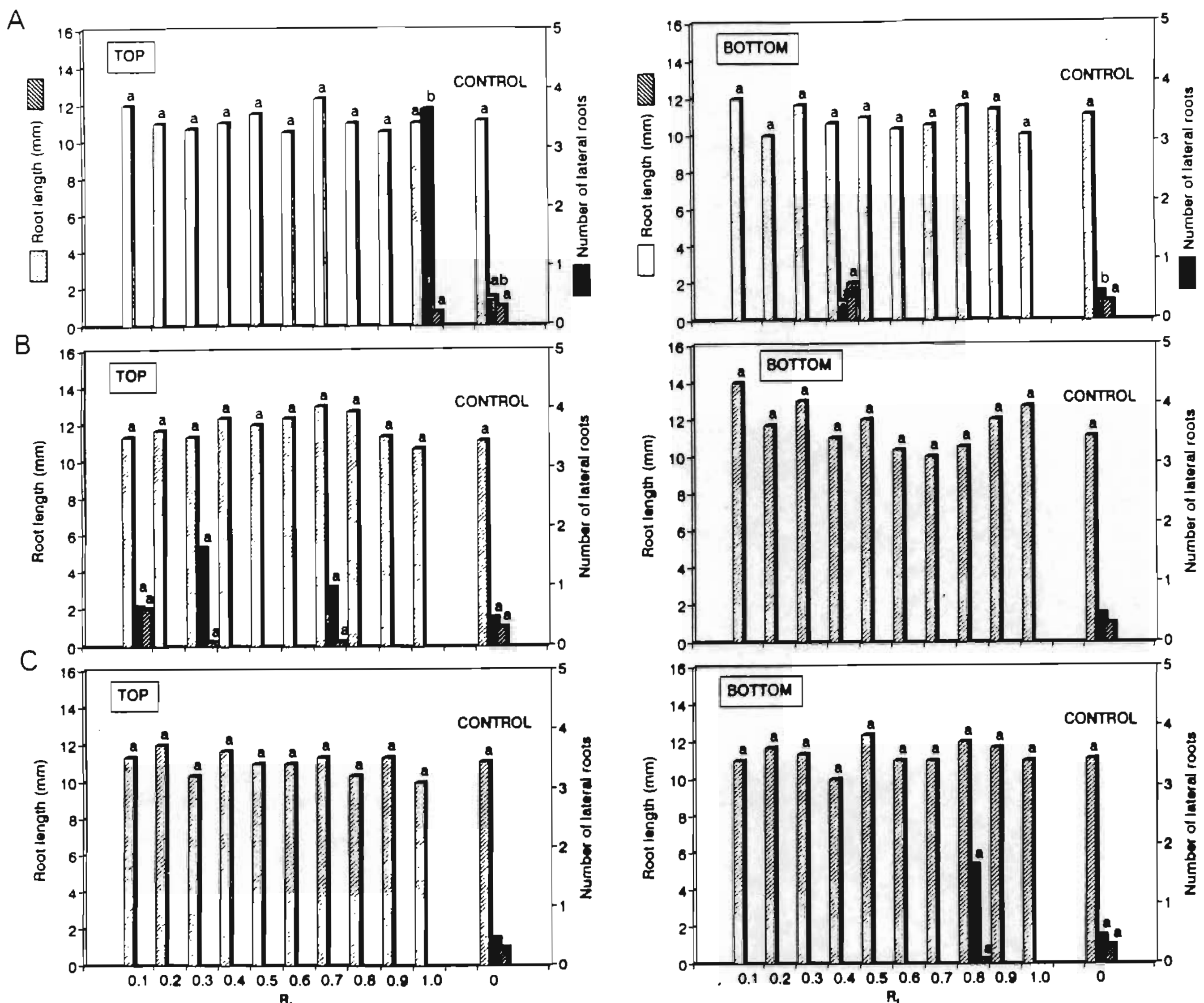


Figure 8.5 The effect of various *Eucalyptus* stem extracts, separated using paper chromatography, on development of *Lycopersicon esculentum* roots grown in suspension culture. Root length (▭); number of lateral roots produced (■) and average length of lateral roots (▨) were recorded. (A) *E.grandis*; (B) *E.macarthuri*; (C) *E.nitens*. Bars bearing different letters are significantly different, $P \leq 0.05$.

8.4 Discussion

The results of this *in vitro* study of the effect of cytokinins seems to indicate that, as previously indicated, high concentrations of cytokinins inhibit root growth. At low concentrations there is, however, a significant stimulative effect. The natural cytokinins used (Z, ZR, DHZ and BA) showed a greater promotive effect than the synthetic cytokinin, kinetin. BA has recently been shown to occur as a cytokinin base, with a limited distribution and abundance in plants (NANDI, LETHAM, PALNI, WONG AND SUMMONS, 1989). Kinetin (0.001 $mg\ell^{-1}$) promoted the growth of two clones of tomato roots on media with high sucrose content (STREET, 1969).

The growth of isolated roots in isolated culture requires practically no auxins. Isolated roots can produce large amounts of auxins (not IAA). Auxin is necessary for division of root cells and for induction of the elongation stage (KEFELI, 1978). Root sections immersed in the culture medium thus can be exposed to supra-optimal concentrations of the hormone. This would explain the poor effect of the applied auxins on root growth (Fig. 8.1 A-C).

Aseptically cultured radicals of tomato (*Lycopersicon esculentum* Mill.) and maize (*Zea mays* L.) accumulate cytokinins, and the developing roots release cytokinins into the culture medium (VAN STADEN AND DAVEY, 1979).

Low concentrations of cytokinins, together with auxins stimulated lateral root formation in contrast to the inhibition by higher concentrations (MACISAAC, SAWHNEY AND POHORECKY, 1989). Too high a concentration of auxin also becomes inhibitory to root growth, but the optimal concentrations of auxin are higher than those of cytokinin by up to one hundred-fold (Fig. 8.1).

The meristematic region of the main root axis appears to inhibit lateral root initiation and growth in close proximity to the root tip. Root decapitation enhanced root branching (STREET, 1969). Roots thus exhibit a similar apical

dominance to that observed in shoots. The main axis is the site of release of an inhibitor (not auxin) that moves basipetally in the root (STREET, 1969), and which is probably a cytokinin.

The results obtained from the application of the R_f fractions from the various stem extracts show that all these fractions, regardless of origin, had little effect on root elongation (Fig. 8.4 and Fig.8.5). With the absence of a dilution series for this experiment, it is possible that the concentration of any promoters and/or inhibitors (including cytokinins) was insufficient to show a result. Lateral root initiation was frequently inhibited, and where initiation did occur, there was no significant increase in root number or length (Fig. 8.5).

In comparison with rapidly growing maize roots, slower elongating roots are more sensitive to the application of zeatin (immersion for 1 hour), without having a higher influx in material. The slower roots grow, the longer the period in which they are affected by immersion in the hormone. Elongation varied according to their initial growth rate (BOURQUIN AND PILET, 1990). This was proposed to be as a result of a differing hormonal balance in the root types. In slow growing roots the concentration of the growth regulator (zeatin) is supra-optimal, and thus inhibits growth. With hormone loss by diffusion into the medium, concentrations decrease enough to allow an initial period of growth, which ceases when synthesis of the growth regulator is resumed. In rapidly growing roots, the hormone concentration is sub-optimal. Initial loss of the hormone to the medium by diffusion, results in a slight decrease in the growth rate followed by a rapid re-equilibration by increasing synthesis. The different reactivity of roots to the application of zeatin could be due to changes in the rate of synthesis or availability of the free growth regulator(s) (BOURQUIN AND PILET, 1990).

The problems that are inherent in the above experiments are the inability to determine the uptake of the hormone by the root segments, and the rate of diffusion or loss of inhibitors and/ promoters into the medium.

CHAPTER 9

CONCLUSIONS

It is evident from the present, and previous research, that the role of cytokinins in adventitious root formation is not simply that of an inhibitor. The complexity of the entire process of adventitious root initiation and development is reflected in the intricate and often confusing roles played by this group of plant growth regulators.

It has been shown that the inability of a cutting to initiate adventitious roots cannot be conclusively linked to high levels of endogenous cytokinins alone. This was illustrated by the similarity in levels of cytokinin-like activity detected in all three species of *Eucalyptus* studied (Chapters 3 and 6). Although the levels of cytokinin-like activity differed between the experiments, the trend across species remained very similar. This could be due to seasonal fluctuations, known to occur in plant growth regulators.

Attempts to alter the cytokinin complement of the various cuttings, and thus improve the rooting potential, met with limited success. Treatment of the cuttings with potassium permanganate did not greatly alter either the cytokinin-like activity detectable in the soybean callus bioassay, or the number and length of adventitious roots produced (Chapter 7). While significant differences were observed between wet and dry centrifugation of the cuttings, centrifugation did not improve the ability of the cutting to produce roots over that of the control (Chapter 6).

It is probable that the physiological processes governing the metabolism of cytokinins are important in the development of adventitious roots. The results presented in Chapter 4 illustrate the quantitative and qualitative changes in cytokinin-like activity over the period of root initiation and development. This

reiterates the necessity of a knowledge of the fundamental biology of rooting before treatments such as hormone applications can be optimised. In this instance, the timing of the hormone application is important.

With respect to treatment of cuttings with exogenously applied hormones, it was found that of the auxins, IBA was particularly effective in root induction, closely followed by the naturally occurring IAA. This latter compound is especially sensitive to light, and enzymatic oxidation (to which synthetic compounds, e.g. NAA, are not subject). The synthetic NAA showed a different trend in root stimulation, with a marked effect on lateral root development (Chapter 5).

The inhibitory role of cytokinins in these experiments (Chapter 5) contrasts with the data obtained for roots grown in suspension culture. In the latter instance the naturally occurring cytokinins, particularly zeatin, zeatin riboside and dihydrozeatin, showed a significant stimulation of root length and lateral root initiation (Chapter 8). This then adds evidence to the theory that cytokinins play a vital role in the development of adventitious roots, i.e. growth in length and lateral root development, while inhibiting the original initiation of root primordia. In this respect the potential of the stem tissues to modify cytokinin levels immediately after striking the cutting, followed by the *de novo* synthesis (in the apical tip of developing root primordia) or the metabolism of existing storage forms to provide optimal levels for root growth, is important in the potential of the cutting to survive and fully regenerate within a short time span (Chapter 2).

It is evident, from the results, that the initiation and development of adventitious roots by cuttings is a highly complex process. Due to this complexity, no single promoter or inhibitor of root induction has been isolated, and the existence of a single limiting rooting morphogen is deemed unlikely, and probably of no relevance (WILSON, 1994). Current research is focusing on the isolation and identification of a number of rooting promoters

(HESS, 1994). It is probable that it is the interaction of these compounds with the various inhibitors present, and the overall physiological status of the cutting, especially prior to striking (Chapter 1), that will determine the success of regeneration.

This method of propagation is of great commercial importance in the horticultural, agricultural and forestry industries. Research into the internal controls of adventitious rooting facilitates comprehension of the fundamental biology of these processes. This in turn enables the efficient manipulation of adventitious root induction and growth.

With respect to research into the controls of adventitious rooting, past physiological and biochemical studies have been aimed mainly at understanding the post-translational processes of rooting on metabolite levels. Although this alone has not led to a sufficient understanding of the control of rooting to enable manipulation of rooting at will, this information can be used to direct future research into the investigation of causal rather than correlative physiology at the gene level. It remains possible that molecular studies at the transcriptional and/or translational levels will reveal the controls of root initiation and development (HAISSIG, DAVIS AND RIEMENSCHNEIDER, 1992).

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APPENDIX

Cytokinin standards (paper chromatography)

Several cytokinin standards (ZG; Z; ZR; DHZ and iP), were prepared (concentration 10 $\mu g \ell^{-1}$) and loaded onto chromatography paper. These were run using the same solvent system as before (Chapter 2.5.2). The R_f fractions from these chromatograms were used as indicators of the cytokinin standards in the soybean callus bioassay, to facilitate comparisons on the basis of co-chromatography. Refer to Figure (i).

Figure (i) Soybean callus bioassays of cytokinin standards separated using paper chromatography. (A) Zeatin glucoside; (B) Zeatin; (C) Zeatin riboside; (D) Dihydrozeatin; (E) Isopentenyladenine.