CHARACTERIZATION AND CONTROL OF MICROPROPAGATION PROBLEMS IN ALOE, DEVIL'S CLAW AND BANANA

By

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Contents

DECLARATION

I hereby declare that, except where acknowledged, the contents of this thesis are the results of my own investigation. The study was done at the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg campus under the supervision of Professor Johannes van Staden and co-supervision of Doctor Wendy A. Stirk.

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MW BAIRU, J NEERU, WA STIRK, and J VAN STADEN. Characterization and control of shoot-tip necrosis in micropropagated *Harpagophytum procumbens*: II - effect of calcium, boron and duration in culture

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LIST OF ABBREVIATIONS

3FBA……. Fluorobenzylamino purine

AFLP……. Amplified fragment length polymorphism

BA……..6-benzylamino purine

B……Boron

Ca……...Calcium

CBP……...Cytokinin binding protein

CDK……...Cycline Dependent Kinase

C/N…… Carbon nitrogen ratio

CTAB……Cetyltrymethyl ammonium bromide

dNTP……Deoxynucleoside triphosphate set

F_v/F_m……..Chlorophyll fluorescence ratio

GA_3…… Gibberellic acid

IAA……Indole-3-acetic acid

IBA……Indole-3-butyric acid

Ip……*iso*-pentenyladenine

Me*m*T……*meta*-Methoxytopolin

Me*m*TR……*meta*- Methoxytopolin riboside

Me*o*T……*ortho*-Methoxytopolin

Me*o*TR……..*ortho*- Methoxytopolin riboside

Me*p*T……*para*-Methoxytopolin

Me*p*TR……..*para*- Methoxytopolin riboside

MS…… Murashige and Skoog (1962) basal medium

*m*T…… *meta*-topolin

*m*TR…… *meta*-topolin riboside

NAA……...ά-Naphthaleneacetic acid

NB……..Nitsch and Nitsch (1969) basal medium

*o*T…… *ortho*-topolin

*o*TR……*ortho*-topolin riboside

PAL……Phenylalanine ammonia-lyase

PCR……...Polymerase chain reaction

PGR…… Plant growth regulator

*p*T…….. *para*-topolin

*p*TR……*para*-topolin riboside

RAPD……. Randomly amplified polymorphic DNA

RFLP……Restriction Fragment Length Polymorphism

STN……...Shoot-tip necrosis

WPM…… Woody plant medium

ABSTRACT

The development of the science of micropropagation from the very initial concept of totipotency to the modern day advancement and sophistication has been affected by a wide range of problems such as hyperhydricity, shoot-tip necrosis and somaclonal variation. These problems are largely the result of the obvious fact of trying to grow plants in an environment that is different from the one plants are used to naturally. The extent of these problems ranges from minor technical inconvenience to significant economic loss. Characterization and control of micropropagation problems has been one of the priorities of plant tissue culture research due to the enormous contribution of this discipline for plant production, improvement and conservation.

The prevalence and severity of these tissue culture problems varies widely among plant species. The rationale of this research project was therefore, to identify plant species most affected by the problems studied, characterize the problem and find mechanism(s) to control or minimize the damage caused by the problem. The literatures reviewed provide sufficient background information for the experimental chapters. Due to the different nature of the problems and variation in the plant species they affect, the model plant, the methodologies used and parameters analysed were also different. The findings of these investigations, in their own different way, addressed certain problems that individually and collectively pose difficulties to the micropropagation industry. The difference in the content of the experimental chapters is therefore the result of the broader objective of the research project to tackle such difficulties.

The success and failure of tissue culture system greatly depends on the choice of PGR's. This choice can be made based on comparative study of their biological activity. Some promising reports on the role of topolins in micropropagation led to the idea of testing these cytokinins for their potential in tissue culture. As a prerequisite to subsequent investigations, the biological activity of some selected topolins and BA derivatives was tested using the soybean callus bioassay. The activity of the cytokinins tested varied significantly. The results demonstrated that

the structure of a cytokinin dictates its activity. Modifications of side-chain improved the activity of *o*T but had no effect on *p*T. The presence of the methyl group had an enhancing effect on cytokinin activity of topolins or at least it did not reduce it. BA derivatives BA_9THP (conjugated at N^9 position), 3FBA and $2Cl_6$(3OHBA)R (halogenated derivatives) also showed good cytokinin activity and hold good promise for future research.

In an attempt to alleviate hyperhydricity in *Aloe polyphylla* and optimize the micropropagation protocol, *meta*-topolin and its derivatives were tested at various concentrations together with BA and zeatin. Of all the cytokinins tested *m*T produced the best results in terms of shoot and root growth. Five µM was found to be the optimum concentration at which complete control of hyperhydricity was achieved without compromising shoot and root growth. Plantlets rooted in a multiplication media. BA generally had a negative effect on growth and development both *in vitro* and *ex vitro*. Acclimatization of plantlets was achieved easily by initially transferring plantlets to a mist house (for three weeks) followed by transfer to the greenhouse. The type of cytokinin also had an effect on *ex vitro* growth with BA-treated plants producing the lowest shoot and root biomass.

Various experiments were conducted to characterize and control factors affecting STN in *Harpagophytum procumbens*. Media type and strength, PGR, carbon sources, sub-culturing, calcium and boron were tested. Results indicated that all of the tissue culture components tested affected STN. From the different media types tested, half strength was MS found to be the preferred medium. Increasing cytokinin concentration increased the incidence of STN and the problem was aggravated by the addition of auxin to the multiplication medium. Optimum shoot multiplication was achieved by omitting auxin and using the cytokinin *m*TR. Plantlets produced basal callus which interfered with rooting. The quantity of this basal callus was minimum when *m*TR was used.

Sub-culturing plantlets onto fresh medium every two weeks helped minimize STN. Off all the sugars tested 3% sucrose was optimum. Other sugars either aggravated STN or inhibited growth when compared at equi-molar concentration. Increasing the concentration of either Ca or B prevented the development of

necrotic shoots. When the concentration of both elements is increased simultaneously negative effects on both growth and STN were observed. Using 6 mM Ca in half strength MS medium was optimum. B was toxic at higher concentrations. Plantlets rooted readily in half strength cytokinin-free MS media supplemented with 2.5 µM IAA. Rooted plantlets produced using the optimized protocol were acclimatized successfully by transferring directly to a greenhouse in a 1:1 ratio of sand and soil mixture.

The effect of *meta*-toplins on micropropagation and somaclonal variation of banana was investigated. Tissue cultured explants of cultivars 'Williams' and 'Grand Naine' were cultured in MS media containing the cytokinins BA, *m*T, Me*m*T, Me*m*TR and *m*TR at various concentrations. Results of the investigation revealed that superior multiplication and lower abnormality index was recorded from the *m*TR and *m*T treatments at 22.2 µM concentration. These treatments, however, had an inhibitory effect on rooting. The effect of these treatments (22.2 µM *m*T and *m*TR) in comparison with equi-molar concentration of BA on somaclonal variation of 'Williams' banana was tested using RAPD-PCR at the 7^{th} multiplication cycle. No significant difference was found between the treatments. It should however be highlighted that cultures were initially maintained for three multiplication cycles in media containing BA. The inherent stability and initial effect of BA could have influenced the results.

1. General introduction and background to the problem

1.1 Introduction

With the fast rate of population growth, human pressure on plants for food and raw material for industry is growing at an alarming rate. This severe pressure has resulted in many species becoming endangered and (in some cases) extinct. The lengthy list of endangered and threatened species presented by the Southern African Botanical Diversity Network (GOLDING, 2002) is an example even though the problem is international. Thousands of plant-derived products across the world originate from the harvest of wild species. The lack of basic knowledge of the ecology of the harvested species, and the absence of production and yield data undoubtedly create further complications to the problem (SHACKLETON and GAMBIZA, 2007). There is a move towards advocating sustainable use of natural resources however, some economic, social and political problems remain as obstacles. PFAB and SCHOLES (2004) stated that “sustainable use of resources” is one of the most commonly misunderstood and misused concepts in contemporary conservation. They further emphasized that without *ex situ* cultivation and farming of these widely used wild resources and addressing human population growth, sustainability can never be achieved.

Mankind has been and is using plants for food, medicinal, horticultural and traditional purposes. Lack of sufficient knowledge of the use of plants coupled with the absence of regulatory mechanisms results in excessive and destructive harvesting of plants. This destructive harvesting in most cases involves the use of whole plants or underground parts that are essential for the survival and reproduction of the plant. In addition to the unsustainable use of plants, urbanization and the development of associated infrastructures causes serious damage to the ecosystem. Due to the change in ecosystems, some plant species such as *Aloe polyphylla* have lost their pollinators; leaving them with little or no chance of natural perpetuation. Furthermore, some species are very sensitive to adult plant harvesting. PFAB and SCHOLES (2004), for example, demonstrated that harvesting only one adult plant annually from a population of 100 *Aloe*

peglerae produces an extinction probability of 100%. This means, harvesting of only 0.12% or less of the mature plants of *A. peglerae* per year can be considered sustainable – virtually impossible to control. These and other facts necessitate the need to develop the heavily used wild plants as farming crops – thereby ensuring their conservation by solving the problems associated with plants being produced commercially.

1.2 Background to the problem

The development and applications of the techniques of plant biotechnology in general and plant tissue culture systems in particular has revolutionized the production, improvement and conservation of plants of economic importance. Rapid shoot multiplication and development followed by acclimatization and establishment of plantlets for field planting is now possible for several species through clonal propagation. The application of these techniques is particularly significant for plants with low regeneration potential due to recalcitrant seeds, problem with pollination and/or specific habitat requirements. The efficiency and use of the technique of micropropagation, however, is greatly affected by associated problems such as hyperhydricity, shoot-tip necrosis and somaclonal variation. These physiological and genetic anomalies of tissue cultured plants can be observed during *in vitro* and greenhouse growth stages.

Comparison of the relative biological activity of some cytokinins, micropropagation and hyperhydricity in spiral aloe (*Aloe polyphylla*), shoot tip necrosis in devil's claw (*Harpagophytum procumbens*) and somaclonal variation in banana (*Musa acuminata*) were investigated in this study. The following subsections of this part of the thesis are presented as separate chapters or sections of this thesis and hence are only intended to give an insight to the forthcoming contents of this research project. They also address the importance of the research project and pose questions relevant to the general objectives of the research.

1.2.1 Comparative study of the biological activity of selected cytokinins using the soybean callus bioassay

Any attempt to develop successful micropropagation protocols depend largely on the precision of the choice of the type and concentration of plant growth substances. This requirement varies between plant species due to their diverse requirements. This diversity between plants makes it a necessity to develop and/or optimize the tissue culture protocols for individual species that are being tissue cultured. The knowledge of the relative biological activity of plant growth regulators (PGR) is therefore, an integral part of this whole process.

VAN STADEN and DREWES (1991) emphasized the importance of bioassays in cytokinin research despite the presence of some sophisticated techniques for the estimation and identification of cytokinins. The selection criterion for any type of bioassay, however, remains largely subjective. The sensitivity of the active substance, morphological variability, and seasonal changes in storage tissues are some of the factors noted to influence selection criteria for bioassays (SKOOG and ARMSTRONG, 1970). According to LETHAM (1967) the relative activities of various cytokinins can be very different and even in reverse order (sometimes) in different bioassays (see chapter 3).

After showing promising results in some tissue culture systems (WERBROUCK *et al*., 1996), topolins have increasingly become the subject of investigations. The soybean callus bioassay employed in this study provides information on the relative biological activities of selected topolins. Apart from the use of these results in the subsequent sections of this project, it provides additional knowledge on these previously unstudied classes of cytokinins. Some previously studied cytokinins are also included as a reference for comparison. Some BA derivatives of great agricultural and horticultural importance are also included (see table 3.1 for description of these cytokinins).

1.2.2 Micropropagation and hyperhydricity in *Aloe polyphylla*

Populations of *Aloe polyphylla* Schonland ex Pillans, commonly called spiral aloe, in its wild habitat have diminished mainly due to threats to its specific habitat requirement, overgrazing (associated with change in water regime), unsustainable harvesting for horticultural and medicinal purposes and increasing rarity of its pollinator, the Malachite Sunbird. As a result, this plant is currently registered in the Red Data List of threatened species by SABONET (TALUKDAR, 2002). Cooperative efforts between conservationists and nurseries to propagate the plant for commercial trade is having some success in reducing the number of spiral aloes collected from its wild habitat (AUBREY, 2002). Attempts have also been made to propagate *A. polyphylla* by tissue culture. However, hyperhydricity of regenerated shoots is proving to be a limitation for tissue culturing of this species (ABRIE and VAN STADEN, 2001; CHUKWUJEKWU *et al*., 2002).

Hyperhydricity of tissue cultured plants has been reported as early as 1960's (HACKETT and ANDERSON, 1967) in carnation cultures. With the increased application of tissue culture, the magnitude of the problem increased from a minor loss to a serious limitation in the use of tissue culture with an estimated loss (sometimes) of more than 50% (ZIV *et al*., 1983). Hyperhydric cultures loose their ability to produce normal shoots and roots, which result in poor multiplication rates of healthy plantlets and failure to acclimatize to *ex-vitro* conditions.

An enormous amount of work has been done on hyperhydricity during the past three decades (HACKETT and ANDERSON, 1967; DEBERGH, 1983; LESHEM, 1983; ZIV *et al*., 1983; Von ARNOLD and ERIKSSON, 1984; GASPAR, 1986; ORLIKOWSKA, 1987; PAQUES and BOXUS, 1987; LESHEM *et al*., 1988; PAQUES, 1991; PIQUERAS *et al*., 2002 and many more). However, failure of total control over the problem coupled with an ever growing need and application of tissue culture to modern science and the emergence of more sophisticated biotechnological practices for plant development, necessitate an investigation towards the control of this problem.

1.2.3 Shoot-tip necrosis in *Harpagophytum procumbens*

Harpagophytum procumbens [(Burch) de Candolle ex], also known as Devil's Claw, is one of the several southern African medicinal plants recently placed in the red data list of endangered species (GOLDING, 2002) because of high demand and subsequent overexploitation in their habitat. It is found in arid areas of Botswana, Namibia and South Africa (CRAVEN and LOOTS, 2002). The tubers of this plant are harvested for the anti-inflammatory properties of the extracts used in the treatment of arthritis, lumbago and muscular pain (VAN HAELEN *et al*., 1983). The total trade of this plant for all southern African countries was ca. 700 tonnes (RAIMONDO and DONALDSON, 2002).

This destructive practice, combined with poor natural regeneration, contributes to gradual depletion of the natural populations of devil's claw. High levels of dormancy and low germination rates (<20%) are characteristics of *H. procumbens*. From the limited number of germinated seeds, few seedlings survive the first year. This necessitates the need for better methods of mass propagation and cultivation in order to satisfy the growing demand of this species. Micropropagation of this valuable plant followed by transfer or cultivation of plants in their natural habitat would be a viable alternative. However, *in vitro* propagation of this species suffers from the serious problem of shoot-tip necrosis (An observation made in the Research Centre for Plant Growth and Development, University of KwaZulu-Natal, South Africa). Characterizing and controlling this problem will, therefore, be a major contribution towards any conservation efforts of this species.

In vitro shoot-tip necrosis (also known as apical necrosis or non-pathogenic dieback) is a common physiological disorder in the micropropagation of many plants. The symptoms result from the senescence and death of tissues in the apical bud, which subsequently proceeds basipetally. There is a transitional zone between the dead apical part and the unaffected lower part of the plant. Lower branches and sometimes the whole plant are affected in severe cases (BARGHCHI and ALDERSON, 1996).

1.2.4 Somaclonal variation in banana

The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of the cells and hence it should, theoretically, not cause variation. Clonal multiplication of genetically uniform plants is the expectation. This expectation is usually considered as the basis for the micro-propagation industry. The occurrence of uncontrolled and random spontaneous variation during the culture process is, therefore, unexpected and undesired (KARP, 1994).

Bananas are one of the most important fruit crops in the world in terms of production and consumption. Cavendish bananas comprise approximately 47% of global banana production and are the most important of all bananas (ARIAS *et al*., 2003). Micropropagation of banana cultivars from the Cavendish subgroup accelerated the production of good quality planting material which in turn transformed the industry by minimizing the time required by farmers to produce planting material conventionally (BAIRU*,* 2004). Despite its huge economic importance, tissue culture of bananas often results in severe genetic defects known as somaclonal variation (LARKIN and SCOWCROFT, 1981). This high rate of somaclonal variation is limiting the use of *in vitro* techniques for banana propagation in large-scale commercial operations and prevents the widespread acceptance of tissue-cultured planting material in the banana industry (SMITH and DREW, 1990a). In banana tissue culture, variation rates less than 5% are commercially acceptable (STOVER, 1987). Therefore, avoiding or reducing the variation rate to the commercially acceptable rate is very important.

1.3 Significance of the study

It is becoming increasingly evident that we can no longer rely completely on nature for plants used in our daily lives. It is therefore, imperative to develop some means of production for both sustainable use and conservation purposes. Plant tissue culture offers a viable alternative in this regard. Tissue culture protocols developed in this study and the findings of the associated problems and their solutions will be

a significant addition to the ever growing knowledge in this discipline of science. The utilization of this knowledge will help reduce the pressure mankind is putting on the plant species studied in this research project and plants in general.

1.4 Objectives of the study

The objectives of this project could be categorized into four main categories

- To compare the relative biological activity of some cytokinins (mainly topolins) using the soybean callus bioassay;
- To optimize the micropropagation protocol of *A. polyphylla* and investigate the use of *m*T and its derivatives as potential replacements to BA and zeatin to improve shoot multiplication and control hyperhydricity;
- To characterize and control shoot tip necrosis in *H. procumbens*, enabling a micropropagation and acclimatization protocol to be developed;
- To investigate the role of *m*T and its derivatives in tissue culture and somaclonal variation of William's banana

2. Literature review

2.1 Cytokinins: biosynthesis, metabolism and activity

2.1.1 Introduction

Different categories of plant growth regulators (PGR's) have been discovered and are available for use. Cytokinins, N^6-substituted adenines with potent plant growth regulatory activity, are among those PGR's that play a major role in plant growth and development. They are defined as 'substances which, in combination with auxin, stimulate cell division in plants and interact with auxin in determining the direction in which differentiation of cells takes place' (McGAW and BURCH, 1995). Due to the diverse effects they induce, cytokinins are considered to be involved in almost all phases of plant development (HORGAN, 1992).

The search for new growth regulatory molecules or compounds is ongoing due to the limitations of the existing phytohormones. Identifying new compounds, their effect and mechanism of action greatly depends on *in vitro* techniques and their applications. *In vitro* culture of plants have been used as closely linked tools in identification and characterization of the role of PGR's in plant growth regulation (KRIKORIAN, 1995).

Since the discovery of cytokinins, the most research has been concentrated on members of the isoprenoid group represented by zeatin, isopentenyladenine, and related compounds (STRNAD, 1997). The aromatic cytokinin BA and its derivatives were considered to be synthetic until the discovery of cytokinins with an aromatic side chain. HORGAN *et al*. (1975) isolated, for the first time, the aromatic cytokinin 6-(2-hydroxybenzylamino)-9-β-D-ribofuranosylpurine from poplar leaves. JONES *et al*. (1996) reported the occurrence of aromatic cytokinins, BA, *meta*-topolins (*m*T) and *ortho*-topolins (*o*T) in various tissues of oil palm (*Elaeis guineensis* Jacq.). STRNAD *et al*. (1997) isolated N^6- (*meta*-hydroxybenzyl) adenine, a highly active aromatic cytokinin, from poplar leaves

(*Populus x canadensis* Moench, cv.*Robusta*) and proposed the trivial name '*meta*-topolin'.

2.1.2 Cytokinin biosynthesis and metabolism

A considerable amount of work has been done on plant hormone physiology. However, the biosynthesis of cytokinins is not fully understood. TAYLOR *et al*. (2003) stated the following factors as obstacles to the understanding of the biosynthetic pathways:

- Extremely low levels of endogenous cytokinins;
- The central role of the likely precursors in cellular metabolism;
- The existence of numerous native substances with more or less pronounced cytokinin activity and;
- The reliance on incorrect or partially correct assumptions about cytokinin biosynthesis.

The absence of a common metabolic pattern for cytokinins makes it difficult to draw a general conclusion about the role of cytokinin metabolites. The improbable condition of all the cytokinins being converted to a common metabolite, which is responsible for growth responses observed, has also been highlighted (FORSYTH and VAN STADEN, 1986). According to PALMER *et al.* (1981) this variation in the metabolism of applied cytokinins could be due to differences between stages of development, physiological condition and plant species analyzed. PALNI *et al*. (1984) noted that while 9-ribosylation of zeatin enhanced activity and O-glucosylation did not significantly reduce it, 7-and 9- glucosylation essentially abolished cytokinin activity.

LETHAM and PALNI (1983) reviewed the biochemical and physiological effects of cytokinins in plants. VENIS (1985) elaborated the structure function relationships of cytokinins and their receptor sites. However, there is no clear cut understanding of the cytokinin action in plants (VAN STADEN and CROUCH, 1996). VENIS (1985) explained that changes in tissue sensitivity could be related to some changes in receptor properties such as their number and/or hormone binding affinity. While emphasizing structure–activity considerations, VENIS (1985)

indicated that the precision of the recognition process can probably only be accommodated in a macromolecular structure and all known hormone receptors are proteins. A detailed study on protein profile and change in polypeptides in response to cytokinin action could, therefore, lead to a better understanding of structure function relations.

Plant tissues metabolize exogenous cytokinins to different types of metabolites such as products of ring substitution (ribosides, nucleotides, N-glucosides) and products of side chain cleavage (adenine, adenosine, adenosine-5'-monophosphate (LETHAM and PALNI, 1983; VAN STADEN and CROUCH, 1996). Although the functional significance of these metabolites is said to be obscure (WAGNER and BECK, 1993), there is a suggestion (LETHAM and PALNI, 1983) that these compounds could be:

1. Active forms of cytokinin, i.e. the molecular form that induces growth or physiological response;
2. Translocation forms;
3. Storage forms which would release free (active) cytokinins when required;
4. Detoxification products formed following exogenous cytokinin application at toxic levels;
5. Deactivation products formed to lower endogenous (active cytokinin) levels; and
6. Post-activation products, formation of which is coupled with cytokinin action.

In an extensive review on benzyladenine and derivatives VAN STADEN and CROUCH (1996) emphasised the absence of a common metabolic pattern when compared to the knowledge on isoprenoid cytokinin metabolism and reviewed the factors that could have contributed to this complexity. The stage of plant development, physiological condition, organ type, plant species used, concentration of supplied compounds and method of application have all been indicated to have an effect on the metabolism of exogenous and endogenous cytokinins. These authors also emphasized the need to determine the active form(s) of the cytokinins.

A study of cytokinin action at the molecular and cellular levels needs to be done in terms of signal perception, transduction and response. This could be performed by looking for proteins which may serve as signal receiving molecules followed by studying the signal transduction pattern(s) (STRNAD, 1997). Very little is known about the molecular mechanism by which target cells for plant hormones translate the signals to specific responses. LIBBENGA and MENNES (1995) stated that in a hormonal system, cells of different tissues and organs not only transmit signals, but they are also capable of detecting signals which they receive from other parts and respond to those signals in their own characteristic way.

In his review STRNAD (1997) explained the specificity and complexity of cytokinin binding. The existence of two related groups of cytokinin binding proteins (CBPs), one with low affinity to zeatin but with strong affinity to BA and the other with an opposite character, indicates that there is a distinction between aromatic and isoprenoid cytokinins with respect to nature of binding and receptor response. The role of modified adenines in cytokinin-binding interaction has been elucidated using X-ray crystallographic structural studies (STRNAD, 1997). IWAMURA *et al*. (1980) however, proposed that differences found in the electron structure and hydrophobicity of N^6-substituent could be due to the cytokinin binding site. The N^6-side chains are undoubtedly important in the binding of cytokinins to their appropriate receptors.

More recently, MOK *et al*. (2005) reported that topolins and hydroxylated thidiazuron derivatives are substrates for cytokinin O-glucosyltransferase with position specificity related to receptor recognition. They demonstrated that the *m*-OH and *o*-OH (*meta* and *ortho* respectively) derivatives are preferred substrates of ZOF1 (enzyme encoded by *P. lunatus*) and *cis*ZOG1 (enzyme encoded by *Zea mays*) respectively. They also noted correlation between the activity of the cytokinins and their ability to serve as a substrate for glucosyltransferase which lead to the conclusion that there may be similarity between cytokinin-binding sites on the enzyme and cytokinin receptors. They found support for their interpretation from cytokinin recognition studies involving the *Arabidopsis* CRE1/WOL/AHK4 and maize ZmHK1 receptors. The AHK4 receptor responded to *trans*-zeatin and *meta*-topolin while the ZmHK1 receptors responded to *cis*-zeatin and *ortho*-topolin.

Cytokinins, in combination with auxins, are also known to affect the basic mechanism of cell proliferation and differentiation. HARRAR *et al.* (2003) demonstrated that this hormonal control of cell proliferation and differentiation requires PASTICCINO (*PAS*) genes. These authors determined the role of the *PAS* gene by analysing the expression profiles of several genes involved in cell division and meristem function and found that differentiated and meristematic cells of the *PAS* mutants were more competent for ectopic cell division, and were especially enhanced by cytokinins. They further demonstrated that disorganised cell divisions were associated with the deregulation of cell cycle marker genes like cyclin-dependent kinase A (CDKA) and cyclin B1 (CYCB1).

2.1.3 Cytokinin activity

The concept of cytokinin structure-activity relationship has attracted the attention of researchers for more than five decades. Now, research interest and focus on the same topic is as fresh as it was earlier due to the growing application of our knowledge of PGRs. It is well documented that the activity of cytokinins is highly affected by their structure; with the structure activity relationship of cytokinins being affected in a number of different ways. LETHAM (1978) reviewed the influence of structural modifications on the activity of natural and synthetic cytokinins and listed the following important features for high activity; an intact adenine moiety with an N^6-substituent of moderate molecular length (SKOOG and ARMSTRONG, 1970), an intact purine ring, unsubstituted 1- and 3- positions, optimum side chain length of five carbon atoms, unsaturation in the side-chain, a substituent which is planar (HECHT *et al*., 1970a; NISHIKAWA *et al*., 1989) and hydrophobic, an electron rich nitrogen group located opposite to the substituent and linkage atoms or a group that connects the purine ring with the side-chain and restrict the molecular configuration (NISHIKAWA *et al*., 1989), 4-hydroxylation of the *iso*-pentenyl side chain (MATSUBARA, 1980) have been mentioned as a structural requirement for high cytokinin activity. CHEN and KRISTOPEIT (1981) on the other hand, suggested that activity depends on the interconversion between the free bases and their ribosides. MATSUBARA (1980) attributed the structure activity relationship of cytokinins to a number of factors which could be placed in

one or more of the above mentioned categories. Of particular interest to this study are the following structural and functional requirements for cytokinin activity. They are discussed briefly: -

i) The general effect of ring substitution of aminopurines

Much of the pioneering work on the effect of ring substitution on cytokinin activity dates back to the 1950s and 60s (for review see MATSUBARA, 1980). Replacing the furfuryl group of kinetin by a wide range of other side-chains was possible without considerable loss of cytokinin activity. BA, arguably the most active synthetic cytokinin owes its discovery to this form of ring substitution – replacing the furfuryl group by a benzyl group. BA was found to be more active than kinetin in the tobacco callus assay. The activity of ring substituted aminopurines is also affected by the degree of saturation. Generally aminopurines with an unsaturated ring are more active than the saturated ones (SKOOG *et al*., 1967; SKOOG and ARMSTRONG, 1970; MATSUBARA, 1980).

ii) The effect of the hydroxyl group on the side chain

Hydroxylation of the *trans* methyl group in the N^6 side-chain of N^6-(Δ^2-*iso*-pentenyl)adenosine increased the biological activity but the activity was not affected or decreased when the *cis* methyl group was hydroxylated (KAMINEK *et al*., 1979). Regiospecific hydroxylation of aromatic cytokinins is believed to regulate their activity (KAMINEK *et al*., 1987a). This form of hydroxylation is likely to have an effect on the dihedral angle and the formation of hydrogen bond with the nitrogen at N^1 position - both known to affect the activity of aromatic cytokinins depending on the position of the hydroxyl group. Taking this fact in to account one would expect *m*T to be more active than *o*T – which is indeed the case (for review see STRNAD, 1997).

The position of the hydroxyl group in the side-chain has a significant effect on the biological activity of the parent cytokinins. Substitution on the phenyl ring enhanced the activity in the order *meta* > *ortho* > *para* in most bioassays for purinyl and urea-type cytokinins (KAMINEK *et al*., 1987a). HORGAN *et al*. (1975)

noted a decrease in activity of N^6-benzyladenosine after hydroxylation of the phenyl ring at the *ortho* position. MOK and MOK (1985) demonstrated that activity increased in order of *meta* > *ortho* > *para* in the *Phaseolus lunatus* callus assay for the hydroxylated derivatives of N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron-TDZ).

iii) The effect of a double bond on the side chain

The presence of a double bond on the side chain is believed to be one of the structural requirements for high cytokinin activity. High cytokinin activity can be achieved or maintained by keeping side-chain planarity. Addition of substituents to the double bond disturbed the side-chain planarity, thereby reducing cytokinin activity (HECHT *et al*., 1970a). The position of the double bond also affects cytokinin activity. LEONARD *et al*. (1968) demonstrated that the 2,3-position of the side-chain as in 6-(3-methyl-2-butenylamino)purine enhanced activity while shifting the double bond to the 3,4-position as in 6-(3-methyl-3-butenylamino)purine reduced the activity in the tobacco callus bioassay. By synthesizing and testing the biological activity of two 6-substituted purines, one with an ά-double bond and one without, NISHIKAWA *et al*. (1986) revealed that the compound with the ά-double bond was twice as active as the one without, indicating the importance of the double bond in cytokinin activity.

iv) The effect of the methyl and amino groups on the side chain

Cytokinin activity is affected by the methyl group(s) and their position on the side-chain of N^6-substituted adenines. Lengthening the bridge or removal of the methyl group between the ring and the amino group on the 6-position of the purine ring of ring substituted adenines decreases cytokinin activity (MATSUBARA, 1980). Disubstitution with two methyl groups at position 3- produced a highly active 6-(γ,γ-dimethylallylamino)purine whereas addition of a methyl group to the 1, 2, or 3-positions of the carbon atom on the side-chain did not affect the activity. However, putting these two methyl groups at position 1- of the side-chain reduced the activity a 100 fold. Shifting the methyl group from position 3- to 2- of the side-chain of *cis* and *trans* zeatin or removing it from *cis* zeatin resulted in a significant

decrease in activity (SKOOG *et al*., 1967; MATSUBARA, 1980). Replacing the amino group of kinetin by a sulphur atom resulted in a considerable decrease in activity suggesting the importance of the amino group on the N^6-position for cytokinin activity (MATSUBARA, 1980).

v) The effect of side chain configuration (geometrical and optical isomers)

The side-chain configuration affects cytokinin activity. The *trans* isomer of zeatin is more active than the *cis* isomer in stimulation of cucumber cotyledon expansion, retention of chlorophyll in detached leaf pieces, induction and stimulation of chlorophyll synthesis in cucumber cotyledon and betacyanin synthesis in *Amaranthus caudatus* seedlings grown in the dark (KAMINEK *et al.,* 1979). The difference in the side-chain configuration of geometrical isomers affects the side-chain planarity, which in turn affects activity (HECHT *et al*., 1970a). The interaction between the cytokinin molecule and its receptor site is also influenced by the absolute configuration around the asymmetric carbon (MATSUBARA, 1980).

vi) The effect of position of the substituent on the purine ring

The position of the substituents in the purine ring affects cytokinin activity. This position of attachment also gives the cytokinins their respective identities. For example 6-benzylaminopurine (BA) indicates that the side-chain is attached on position 6 of the purine ring. Much research has been done on the position effect of the substituent across the purine ring (For review see MATSUBARA, 1980). Substitution at N^1 lowered activity but the activity was enhanced after the compound was autoclaved. The assumption was made that autoclaving might have helped the conversion to the active N^6 isomers. Further investigations, however, suggested that the reactive N^1 position must remain free to make the cytokinin active (MATSUBARA, 1980). This concept further elaborates the fact that *o*T is less active than *m*T due to the formation of the hydrogen bond with the nitrogen at N^1 position (see part **ii** of this section).

The effect of substitution at N^2 varied depending on the type of assay and the nature of the substituents. An example of a compound with substitution at N^3

position is triacanthine – a naturally occurring adenine derivative. Like the isomers with N^1 substitution, triacanthine was active only following autoclaving leading to the suggestion of producing N^6-benzyladenine (LEONARD and HENDERSON, 1975). These authors demonstrated that when autoclaved, 3-substituted adenines undergo rearrangement and convert in low quantity to N^6-substituted adenines (BA). N^6 substituted adenines are the most active of all N-substituted adenines and generally the monosubstituted ones are more active than their corresponding disubstituted (SKOOG *et al*., 1967). Substitutions at positions N^7, N^8 and N^9 produce inactive or slightly active, depending on the nature of the substituent, molecules (MATSUBARA, 1980).

vii) The effect of halogen substituents

The addition of electron withdrawing and hydrophobic substituents improved the activity of phenylurea cytokinins (RICCI *et al*., 2005). This however, was greatly affected by the position of substitution and number of substituents. For instance monosubstitution with Cl or Br at *ortho*, *para* or *meta* positions on the phenyl ring decreased activity though the decrease caused by *meta* substitution was minimal. Substitution at the 2-position of the pyridyl ring with halogens increased activity as opposed to substitution with CH_3, OH or NH_2 which did not affect the activity (SHUDO, 1994).

The activity enhancing effect of halogen substitution on isoprenoid (CLEMENCEAU *et al*., 1996; HAIDOUNE *et al*., 1998) and aromatic (DOLEŽAL *et al*., 2006, 2007a) cytokinins was also reported. Different authors explained the activity enhancing effect of halogen substitution differently, among others: high electronegativity and small size of the halogens (CROCKER *et al*., 2007), the ability of organo-halogen compounds to form structural motifs via inter-molecular interactions (EMMERLING *et al*., 2007), decrease sensitivity of the compounds to cytokinin oxidase (CLEMENCEAU *et al*., 1996; HAIDOUNE *et al*., 1998), and forming an hydrogen bond with electron donors of a cytokinin receptor (DOLEŽAL *et al*., 2007a) are thought to be contributing factors.

The structural variability of organic compounds in general and cytokinins in particular offered a wide range of possibilities to modify their structure and

enhance their application spectrum. The variations in the biological activity of the same cytokinins in various bioassay systems are logical indications of the diverse recognition patterns and/or signalling mechanisms that may operate in cytokinin-dependent physiological responses. It also highlights the possibility of designing specific compounds to modulate a particular cytokinin-dependent response (DOLEŽAL *et al*., 2006; DOLEŽAL *et al*., 2007a). This makes interpretation of biological activity test results relative to the nature of the assay and plant material used.

2.2 Hyperhydricity

2.2.1 Introduction

A variety of terminologies has been used to describe this abnormal physiological and morphological condition. These include; hyperhydricity, translucency, hyperhydration, succulency and glassiness (ZIV, 1991). The most often used term to describe such abnormalities *in vitro* is vitrification. This term however, is said to be misused as it refers to physical and not biological processes (ZIV, 1991). These abnormalities have been redefined as hyperhydricity (DEBERGH, *et al*., 1992). High relative humidity, poor gaseous exchange between the internal atmosphere of the culture vessel and its environment, and the accumulation of ethylene that may induce physiological disorders, are characteristic features of the tissue culture environment. Given the importance of optimum culture conditions for successful clonal propagation, it is imperative to undergo an investigation towards the type and cause of the abnormalities induced by these culture conditions.

2.2.2 Definition and cause of hyperhydricity

Hyperhydricity is defined as the morphological and physiological disorder of plants grown *in vitro*, which results in a loss of ability to grow normally and impede *in situ* acclimatization (PAQUES and BOXUS, 1987). Hyperhydricity can be of two types, one resulting from the passive diffusion of water into tissues and the other from an active phenomenon related to a disturbance in metabolic processes (PAQUES, 1991). Possible causes of hyperhydricity (GASPAR *et al.,* 1987) include

environmental factors, gelling agents, growth regulators and others. Hyperhydricity is a complex response and tissues having this problem show a wide variety of side effects namely, oxidative stress, anoxia and mineral deficiencies, alteration of peroxidase and failure in lignin biosynthesis (PIQUERAS *et al*., 2002). Some of these factors are discussed below.

i) Plant growth regulators

Plant growth regulators are among the most frequently reported causes of hyperhydricity (ORLIKOWSKA, 1987; LESHEM *et al*., 1988). PARK *et al*. (2004) reported that application of an ethylene scrubber (perlite treated with $KMnO_4$) resulted in the normal growth of potato shoots without any sign of hyperhydricity. They also found no ethylene in a completely sealed vessel in the presence of the ethylene scrubber. GASPAR (1986) explained the possible role of ethylene on hyperhydricity and suggested an hypothetical sequence of reactions leading to hyperhydricity. He suggested that there is an increase in peroxidases and high initial production of ethylene during the first few days due to stress conditions in culture. This and other culture conditions impede lignification and encourage increased water uptake.

TURGEON (1982) compared abnormal plants in culture with Crown Gall teratomata having many short stems and irregularly arranged thick leaves. These teratomata led to abnormal synthesis of auxins and cytokinins (AMASINO and MILLER, 1982). LESHEM and SACHS (1985) also suspected a possible change in the synthesis of auxin and cytokinin when shoot tips are excised and cultured *in vitro*. Results of these investigations indicated that a high concentration of NAA increased the proportion of hyperhydric plants. The effect was pronounced in the absence or low concentrations of cytokinins and resulted in disorganised development and callus formation. Cytokinins counteracted the effect of high auxin. During sub-culturing, only shoot tips from abnormal plants were able to grow in the absence of any auxin. LESHEM and SACHS (1985) suggested this to be due to growth factor(s) imbalance. At equivalent concentration NAA caused more abnormality than IAA.

Reports indicate that the cytokinin BA causes hyperhydricity *in vitro* (LESHEM and SACHS, 1985; LESHEM *et al*., 1988; TERAMOTO *et al*., 1993). LESHEM *et al*. (1988) demonstrated that there was no hyperhydricity in the absence of BA in melon cultures, but 0.01 mg l^{-1} BA was enough to induce 100% hyperhydricity. The auxin indoleacetic acid alone did not cause hyperhydricity. They further noted that none of the other accepted factors inducing hyperhydricity such as a low agar concentration, high relative humidity and a high concentration of ammonium caused hyperhydricity without the presence of BA in the medium.

ii) Agar quality and concentration

Different reports indicate that it is possible to reduce the incidence of hyperhydricity, with a negative impact on shoot multiplication and rooting, by increasing the concentration of agar (DEBERGH, 1983; LESHEM, 1983; ZIV *et al*., 1983; Von ARNOLD and ERIKSSON, 1984; ORLIKOWSKA, 1987). Both the concentration and brand of agar affected the chemical and physical characteristics of the culture medium (DEBERGH, 1983). He also noted that the availability of labelled kinetin was decreased with an increased concentration (8-15 g) of Difco Bacto agar in tissue cultured globe artichoke. The concentration of Difco Bacto agar was found to have a significant effect on the levels of Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, and Mn^{2+}; the higher the agar concentration the higher the levels of these ions. This was also related to impurities introduced with the agar (DEBERGH, 1983).

BORNMAN and VOGELMANN (1984) reported a significant inverse correlation between radiolabelled N^{6}-benzyladenine (^{14}C-BA) accumulation and degree of gel stiffness for both agar (Tayio) and none-agar (Gelrite) media. They found significantly greater numbers of adventitious buds induced on media at low to medium levels of rigidity. However, at extremely low concentrations of gelling agents (<5.0 g Tayio l^{-1} and 1 and 1.5 g Gelrite l^{-1}), a high level of hyperhydricity nullified the results. BROWN *et al*. (1979) reported that the water potential of the medium was highly influenced by agar.

iii) Relative humidity and ventilation

PARK *et al*. (2004) investigated the effect of ventilation on hyperhydricity of potato shoots with and without the ethylene inhibitor $KMnO_4$ and found that shoots grown in a gas permeable vessel were normal as opposed to shoots grown in completely sealed vessels. They also indicated that the levels of ethylene and CO_2 were significantly higher in the completely sealed vessels and little ethylene was detected in the gas permeable vessels. ZIV *et al*. (1983) found that lowering the relative humidity of the medium helped reduce hyperhydricity. GRIBBLE (1999) stated the possibility of manipulating the relative humidity of the culture media to produce plantlets of a particular morphology.

iv) Light and temperature

Optimum light and temperature are among the critical requirements of clonal propagation. Hyperhydricity could be reversed (up to 80%) by lowering the temperature of the culture medium by means of a bottom cooling device with no additional change in other culture conditions (PIQUERAS *et al*., 2002). They also indicated that spontaneous reversions of hyperhydricity never exceed 20%. GRIBBLE (1999) argued that the efficiency of a bottom cooling device could easily be affected by small changes in the *in vitro* temperature distribution. The placement of culture vessels in relation to light is one of the factors that could bring a change in culture temperature.

v) Organic and inorganic components

A variety of organic and inorganic components play a role in the normal growth and development of a plant and factors governing it. Any irregularity in the production, function and uptake of these components is likely to affect the overall physiological activities of the plant. In tissue culture, plants are exposed to an artificial supply of organic and inorganic compounds that could upset the natural balance. Different reports, for example, indicate that the large quantities of ammonium ions in tissue culture increase hyperhydricity by causing toxification to the activities of some enzymes involved in the synthesis of lignin and by lowering

the C/N ratio of the media (KEVERS *et al*., 1984; VIEITEZ *et al*., 1985; DAGUIN and LETOUZE, 1986; PHAN and HEGEDUS, 1986).

2.2.3 Physiological and biochemical reactions leading to hyperhydricity

DEBERGH *et al*. (1981) stated that the source and the physiological stage of the mother plants at the time of culture initiation do not interfere with hyperhydricity. KEVERS *et al*. (1984) made a comparative study of the physiological and biochemical events leading to hyperhydricity. Water content, peroxidase activities and isoperoxidases, phenylalanine ammonia-lyase (PAL) and phenolic contents were comparatively analysed in tissue of normal and hyperhydric plants of different species cultured *in vitro*. Results of these investigations indicated that hyperhydricity results from a burst in ethylene controlled by the peroxidase-IAA-oxidase system. There was considerably higher soluble peroxidase activity in hyperhydric plants. Electophoretic zymogrames of all analysed plants uniformly showed a general increase in the activity of basic isoenzymes and a decrease in the activity of acidic ones. PAL activity and phenol content were consistently lower in hyperhydric plants (KEVERS *et al*., 1984).

i) Water content

Hyperhydricity is highly influenced by the total water potential of the medium and the availability of water (BROWN *et al*., 1979). VIEITEZ *et al*. (1985); WERKER and LESHEM (1987) explained cell hypertrophy observed in hyperhydric plantlets in terms of an enhanced diffusion of water into the cell. Hyperhydricity in plant tissue culture has been associated with reduced transpiration and increased water uptake. This phenomenon plays a role in reducing the total level of oxygen within plant tissues, which in turn leads to other biochemical disorders (PIQUERAS *et al*., 2002). Comparative analysis of dry weight percentage in normal and hyperhydric plants of seven species was compared and resulted in a 0-43% lower dry weight and a correspondingly higher fresh weight in hyperhydric samples (KEVERS *et al*., 1984).

ii) Lignification and cell wall components

It is believed that in initial cultures stress caused by excess PGR's (mainly cytokinins) or NH_4^+ enhances the activity of soluble and membrane-bound peroxidase through a rapid modification of the phenolic level (KEVERS *et al.*, 1984; GASPAR *et al*., 1987). Phenylalanine ammonia-lyase (PAL) and acidic peroxidase are involved in the process of cell wall lignification (EGLEY *et al*., 1983; GOLDBERG *et al*., 1983). The excess of ethylene in the atmosphere of stressed plants inhibits its own biosynthesis. This causes a decrease in the activities of PAL and acidic peroxidase which in turn impedes the lignification processes (KEVERS *et al*., 1984).

The biosynthesis of lignin and cellulose are closely associated (KEVERS *et al.,* 1984). A lower C/N ratio that is caused by an excess of nitrogen, results in the deficiency of both lignin and cellulose. Lignin, in addition to cellulose, plays a significant role in cell wall rigidity. Deficiency of both lignin and cellulose would allow greater water uptake due to reduced wall pressure. This results in hyperhydricity.

iii) Chlorophyll and photosynthesis

Lower photosynthetic efficiency is a characteristic feature of *in vitro* plants. Hyperhydric plants have less chlorophyll than normal plants. The ratio chlorophyll a/chlorophyll b, however, remains the same (ZIV *et al*., 1983). LESHEM (1983) however, indicated that the photosynthetic rate of hyperhydric plants is about half that of normal plants.

2.2.4 Ultrastractural aspects of hyperhydricity

Studies using scanning electron microscopy (WERKER and LESHEM, 1988) revealed that thickening of the stem and retardation of elongation are the first changes observed in hyperhydric carnation plantlets. More recently, OLMOS and HELLIN (1998), made a comparative study of ultrastractural differences of hyperhydric and normal leaves of carnation plantlets using scanning and

transmission electron microscopy. Their results indicated that large vacuolated mesophyll cells (showing hypertrophy of cells and large intercellular spaces), lack of cuticular wax and presence of abundant plastoglobuli on chloroplasts were characteristic features of hyperhydric leaves. They also noted differences in the morphology of guard cells with X-ray microanalysis revealing high levels of K^+ on abnormal plants. Stomatal density was significantly greater in normal leaves with the crystalline structure of the epicuticular wax absent in hyperhydric leaves. An irregular assortment of organelles and unorganised spongy mesophyll were also observed in hyperhydric leaves.

There are reports which indicate that hyperhydricity is a reversible process (PIQUERAS *et al*., 2002). OLMOS and HELLIN (1998), however, suggested the possibility of this being an ontogenic process. DELARUE *et al*. (1997) also isolated a new class of recessive *Arabidopsis* mutant that display genetically heritable hyperhydric symptoms such as translucent and wrinkled cotyledons and leaves, abnormal chloroplast organization, a reduced amount of chlorophyll, a reduced dry weight and a decreased number of palisade cells. They mapped the recessive gene (*cristal* or *cri1*) on chromosome 4 close to the DHS1 marker. These findings trigger the need for molecular studies of hyperhydricity that involves the nucleic acids.

2.3 Shoot-tip necrosis (STN)

2.3.1 Introduction

At the first glance of the symptom of STN, browning of buds and youngest leaves, one would automatically assume the cause to be nutrient deficiency. This is because of the general understanding that symptoms of nutrient deficiency of less mobile minerals such as Ca and B (RAVEN, 1977) first appear on the meristematic regions and young leaves whereas symptoms of excessive presence of these minerals are observed on the older leaves (BARGHCHI and ALDERSON, 1996). STN *in vitro*, however, is the function of a complex set of factors. PIAGNANI *et al*. (1996), for example, suggested that in addition to Ca, the

involvement of a complex set of other factors in STN could have contributed to the discrepancies found by different authors. They, however, stated that hormonal balance could be of greater importance.

2.3.2 Factors contributing to shoot necrosis in plants

The problem of non-pathogenic dieback or shoot-tip (apical) necrosis *in vitro* has been associated with various culture conditions such as salt formulation and growth regulators (VIEITEZ *et al.,* 1989; KATAEVA *et al.,* 1991; MACKAY *et al.,* 1995; PIAGNANI *et al*., 1996; GRIGORIADOU *et al.,* 2000; PEREZ-TORNERO and BURGOS, 2000), use of activated charcoal (MACKAY *et al.*, 1995; WANG and VAN STADEN 2001), duration in culture (GRIGORIADOU *et al.,* 2000), type of tissue culture medium (GENEVE *et al*., 1992; MACKAY *et al.,* 1995; GRIGORIADOU *et al.,* 2000), exudation of phenolics in tree species (BELLAROSA, 1988), physiological disorder associated with rooting (KATAEVA *et al.,* 1991), sulphur content and NH_4/NO_3 ratio (LAKSHMI and RAGHAVA, 1993), fructose (KULKARNI and D'SOUZA, 2000) and pH fluctuations (De BLOCK, 1990). These are discussed in detail below.

i) The role of plant growth regulators

PIAGNANI *et al*. (1996) cited the main cause of STN to be the general tendency of most protocols to leave or reduce the quantity of cytokinins to a very low level because of their anti-rooting activity. MACKAY *et al*., (1995) reported that plants cultured on a medium supplemented with iP or kinetin showed excessive yellowing of leaves and necrosis at the higher concentrations. At higher concentration TDZ also showed similar results. GRIGORIADOU *et al.*, (2000) on the other hand reported that BA has no effect on shoot tip necrosis of the pear cultivars 'William's' and 'Highland' grown *in vitro*. However, they indicated that STN increased with BA concentration in *Quercus ruber* emphasizing that response to BA could be highly genotype dependent. PEREZ-TORNERO and BURGOS (2000) found that dipping the shoot tips in BA solution prior to transfer to rooting medium helped overcome apical necrosis in cultures of apricot cultivars; a finding similar to that of PIAGNANI *et al*., (1996) where BA was used to control STN of chestnut *in vitro*.

Unlike reports on the different effect of cytokinin on STN, the effect of auxins on STN is more consistent. According to PEREZ *et al*. (1985), IBA added to the rooting media was responsible for the apex degeneration of *Corylus avellana* shoots. BARGHCHI and ALDERSON (1996) highlighted the possibility of auxins added to culture media altering the production or availability of endogenous cytokinins which could have prevented STN. This argument could also apply for other growth substances or requirements. PIAGNANI *et al*. (1996), for example, showed that Ca treatments which prevent necrosis significantly affected rooting. This result could suggest a possible role of Ca on auxin activity and/or availability. It has long been reported that Ca mediates the effect of auxins and cytokinins (for review see HEPLER and WAYNE, 1985; HEPLER, 2005; HIRSCHI, 2004).

ii) The role of calcium

Calcium is a relatively large essential divalent cation found in large quantities in plant tissues. In contrast to other macronutrients, a high proportion of total calcium is present in the cell walls (some associated with the cell wall and some exchangeable at the plasma membrane). Calcium can also be found in significant quantities in the vacuole. Without causing harmful effects to plant growth, calcium could represent as high as 10% of the total dry weight in some plant species. What is interesting though is despite the large concentration of cellular calcium, the cytosolic-free Ca^{2+} activity within cells remains around the 0.1 to 0.2 µM level (MARSCHNER, 1995; HIRSCHI, 2004). It is believed that this low intracellular concentration gives calcium an advantage over the other abundant cations to act as a carrier of information (HEPLER and WAYNE, 1985 and references therein; ZOCCHI and MIGNANI, 1995).

In his well-written review, HEPLER (2005) elaborated the multiple roles Ca^{2+} plays in plants and emphasized the crucial nature of calcium as a regulator of plant growth and development. HIRSCHI (2004) discussed that versatility and specificity are two contrasting roles Ca^{2+} plays in plants. This is mainly due to the fact that plants rely on the unique properties of Ca^{2+} for their structural, enzymatic and signalling functions. Calcium is involved in cell elongation and cell division,

influences the pH of the cells in addition to its role as a regulatory ion in the source-sink translocation of carbohydrates. Ca^{2+} strengthens cell walls and provides stress tolerance (biotic and abiotic). This is why calcium deficiency in plants, in most cases, is associated with diseases and post harvest problems. It stabilizes cell membranes by connecting various proteins and lipids at membrane surfaces (MARSCHNER, 1995; HIRSCHI, 2004). DYSON and DIGBY (1975) also indicated the importance of Ca in maintaining the apical dominance of potato sprouts and to prevent some of the changes which could attribute to physiological aging.

Ca^{2+} deficiency also causes serious damage to plant cell ultrastructures. In plants deficient in calcium, the nuclear envelope, the plasma and vacuolar membranes deteriorate and structure-less areas appear in the cell resulting in the disorganization of other structures such as the golgi apparatus, mitochondria and plastids (SHA *et al*., 1985). This disorganization in turn results in rapid deterioration of metabolically active tissues such as the shoot apices of shoot cultures resulting in STN.

There are many reports on the role of Ca in shoot-tip necrosis both *in vitro* and *ex vitro*. These reports however, lack consistency as some report that the use of higher concentrations of calcium alleviates or controls the problem (BARGHCHI and ALDERSON, 1996; CHANG and MILLER, 2005; DYSON and DIGBY, 1975; MARTIN *et al*., 2007; PIAGNANI *et al*., 1996; SINGHA *et al*., 1990; VIEITEZ *et al.,* 1989; WANG and VAN STADEN, 2001). GRIGORIADOU *et al.*, (2000) on the other hand reported that high calcium concentrations in culture media significantly increased the percentage of STN. BHALLA and MULWA (2003) also reported that increasing Ca concentration in macadamia rooting experiments failed to stop STN; concentrations more than 6 mM even aggravated the problem. This is opposite to *ex vitro* plant growth conditions where calcium deficiency causes symptoms like leaf-tip burn and blossom-end rot in fruits (SHEAR, 1975). In plant tissue culture media, it is also known that calcium and boron deficiency may cause STN and death of shoot-tip meristems respectively. Although many reports support the association between low Ca levels and shoot necrosis, the effect of Ca could vary from species to species.

PIAGNANI *et al*. (1996) and WANG and VAN STADEN (2001) demonstrated that the addition of Ca to the media alleviated the problem of STN *in vitro* in chestnut and tree peonies respectively. The former however, indicated that the effect of calcium was indirectly due to its role as a mediator of hormone activity. They also demonstrated that the genotype with higher affinity for calcium was less sensitive to STN. Genotype dependent response for both ion uptake and translocation was indicated as Ca appear to accumulate at the basal and middle part of the shoot with a very slow movement to the apex which possibly causes Ca starvation to the shoot-tip (PIAGNANI *et al*., 1996).

BARGHCHI and ALDERSON (1996) demonstrated the effects of the form in which calcium is supplied in *Pistacia vera* cultures. Higher concentrations of calcium supplied in the form of calcium chloride reduced STN and had no inhibitory effect on shoot multiplication or elongation whereas calcium supplied in the form of calcium acetate had severe inhibitory effects on shoot elongation. BORCHERT (1986) reported that calcium absorption by *Gleditsia* leaf tissue was affected by the form in which calcium was applied. He noted that calcium absorption was very fast when applied as calcium acetate as opposed to calcium chloride. The inhibitory effect of calcium acetate, according to BARGHCHI and ALDERSON (1996), could therefore be due to excessive absorption of calcium or a resultant change in the pH of the medium.

iii) The role of boron

Boron requirements differ widely among plant species and it is known to have a narrow range between deficiency and toxicity when compared to other mineral nutrients (ABDULNOUR *et al*., 2000). In addition, what is optimum for one species could be toxic for another (BARGHCHI and ALDERSON, 1996). HU and BROWN (1994) provided cytological evidence for the structural role of boron in the cell wall. They also noted that one of the effects of boron deficiency was a rapid inhibition of growth of root and shoot meristems; an observation that underlines the importance of boron for growing tissues. Boron toxicity symptoms on the other hand occur on the margins of old leaves (SHELP *et al*., 1995). These observations for many years led to the general belief that boron is an immobile element; an

understanding that has now been changed. In their review BROWN and SHELP (1997) classified plants in two categories based on their B mobility; those with restricted B mobility and those in which B is highly mobile. OERTLI (1993), in an experiment made on tomato plants, showed that little boron was remobilized and transported to plant tops while small but adequate amounts were remobilized and transported to the roots. Apart from the normal transpiration stream, boron transport and remobilization involves a number of other factors. Among others the effect of sugar alcohols on this microelement is discussed below.

BROWN and HU (1996) demonstrated that phloem mobility of boron is species dependent. Their investigation revealed that in sorbitol-rich species (*Pyrus, Malus* and *Prunus*) boron is freely mobile as opposed to sorbitol-poor Pistachio – known to have the problem of STN (BARGHCHI and ALDERSON, 1996) and where boron is largely immobile. They suggested that in such species boron mobility is mediated by the formation and transport of B-sorbitol complexes. By isolating and characterizing B-sugar alcohol complexes HU *et al*., (1997) underlined that the main factor that confers phloem boron mobility in plants is the synthesis of sugar alcohols and the subsequent transport of the B-sugar alcohol complexes.

According to BROWN *et al*. (1999) tobacco plants, genetically engineered to synthesize sorbitol, showed a marked increase in within-plant boron mobility and increased growth and yield when grown with limited or interrupted soil boron supply in contrast to their wild counterparts (the controls). They further elaborated that growth of these transgenic tobacco plants was maintained by reutilization of boron present in matured leaves as opposed to the controls. Using isotopic boron, they verified that boron was phloem mobile in the transgenic lines (have sorbitol) but immobile in the control lines. LEHTO *et al*., (2000) reported that boron is re-translocated in some species as a B-sugar alcohol complex. It was suggested that the B-manitol and B-pinitol complexes serve as a possible means of transport.

iv) Association between Ca, B and PGR

Ex vitro experiments on boron fertilization (WOJCIK and WOJCIK, 2003) showed some association between boron and calcium in plant metabolism. Foliar boron

application on pear trees increased calcium in fruitlets, fruits and leaves but showed no effect on nitrogen, phosphorus, potassium and magnesium in plant tissues. ABDULNOUR *et al*., (2000) studied the effect of boron on Ca uptake in micropropagated potatoes and found that excess boron (0.1 and 0.3 mM) could adversely affect calcium uptake. Media with high boron levels decreased the Ca content in shoots and leaves but media with a boron content of 0.025 mM, four times less than the control MS, enhanced calcium uptake. REDONDO-NIETO *et al*., (2003) demonstrated the relationship between boron and calcium in nitrogen fixation. High calcium enhanced tissue invasion by *Rhizobium* was inhibited by boron deficiency and increased boron concentration in the nodules of plants grown in boron free media, suggesting that calcium promoted boron import to the nodules. One could therefore assume that the role of these factors on the problem of STN *in vitro* could be due to the effect they exert on one another.

BOHNSACK and ALBERT (1977) demonstrated that boron deficiency increased the level and activity of IAA oxidase of squash root tips thereby inhibiting cell elongation. This in turn, could result in the total or partial disruption of some physiological processes related to rooting. In their review HEPLER and WAYNE (1985) outlined the role Ca plays in mediating cytokinin action. Ca deficiency can apparently block mitosis due to its effect on cytokinins which are involved in mitotic-cytokinetic processes.

v) The role of physiological processes associated with rooting

Contrary to reports by GRIGORIADOU *et al.* (2000), VIEITEZ *et al.*, (1989) indicated that calcium deficiency, lack of cytokinins and presence of auxins caused STN in European chestnuts and oaks during the rooting stage. KATAEVA *et al*., (1991) elaborated on this report by associating STN with rooting. They explained that cultivation of micro shoots of apple varieties with low rooting ability in cytokinin-free medium leads to depletion of cytokinin from the shoots. This means that in the absence of roots, shoots had no source of endogenous cytokinin. This in turn led to cellular necrosis due to termination of cell division in the apical meristems. XING *et al*., (1997) overcame necrosis by using a calcium-rich rooting medium with reduced cytokinin and no auxin for the American chestnut. LAKSHMI

and RAGHAVA (1993) on the other hand reported that doubling calcium strength in the medium did not help prevent STN in rosewood culture. They controlled the disorder by using a sulphur enriched medium and modifying NH_4/NO_3 ratios. PIAGNANI *et al*. (1996) on the other hand found that various levels of NH_4^+, K^+ and SO_4^- had no significant effect.

STN, however, is not always associated with the rooting phase and occurs even at shoot induction stages. GRIGORIADOU *et al*., (2000) noted STN during the first week of culture initiation and the severity increased with increased duration in culture. ABOUSALIM and MANTELL (1994) noted STN in potato and *Pistacio* cultures during the multiplication stage which was linked with calcium deficiency. KULKARNI and D'SOUZA (2000) also reported that STN occurred during shoot induction in *Butea.* They controlled it by supplementing the media with 10 mg l^{-1} fructose.

vi) Effect of aeration

Tissue culture systems generally involve the use of tightly closed culture vessels to prevent evaporation, desiccation of tissues and to keep cultures sterile. These tightly closed culture vessels however, create some physical and chemical conditions that make normal developmental processes difficult. The first of these is the high relative humidity in the culture vessels. This reduces the transpiration stream thereby affecting the efficiency of transport and translocation of nutrients (SHA *et al*., 1985). Accumulation of some gases such as CO_2 and ethylene is another feature of sealed culture vessels (DUNWELL, 1979). SHA *et al*. (1985) elaborated that the accumulation of these gases could lead to some physiological disorders such as reduced respiration rates, low or abnormal metabolic activities, and an accumulation of some metabolites like amino acids and oxalates. These metabolites in turn, could bind with some important elements like Ca; making it unavailable to the plant.

BHALLA and MULWA (2003) reported that STN in macadamia was linked with inadequate aeration of the cultures. OGASAWARA (2003) discussed the effects of ventilation and noted that ventilation conditions affected the water-vapour pressure

and concentration of organic volatile substances such as ethylene and CO_2 inside the culture vessel. This effect on volatile substances in turn affected the physiology (including the translocation of photosynthates and essential elements) and morphology of plants (OGASAWARA, 2003 and references therein). MARIN (2003) indicated that exposing plants to low relative humidity before transplanting *ex vitro* improved survival rate of acclimatized plants. This effect could be by triggering some changes (functional and structural) that helped the plant become autonomous. The deposition of more crystalline epicuticular wax on the leaf surface of plants (BENZIONI *et al*., 2003) is one of the advantages ventilated plants have over non-ventilated ones.

vii) Other factors

MACKAY *et al*. (1995) reported that the addition of activated charcoal reduced or eliminated chlorosis and callus growth in Mexican redbud (*Cercis canadensis var. mexicana*) regardless of the gelling agent used. MARTIN *et al*., (2007) reported that timing and frequency of shoot necrosis in micropropagated bananas was influenced by the cultivar after a varying number of sub-cultures. PIAGNANI *et al*. (1996) also reported genotype dependent response to treatments against STN. According to PIAGNANI *et al*. (1996) this difference in genotype could be due to the difference in the ability of plants to absorb and translocate Ca, inherent difference in Ca requirement and difference in Ca transport at the membrane level. Increasing the agar and Ca concentration of the media were also reported to have positive effect on STN and vitrification of quince cultures up to a certain level whereafter growth was retarded (SINGHA *et al*., 1990).

2.4 Somaclonal variation

2.4.1 Introduction

Bananas, staple food and important source of carbohydrate for millions of people in the tropical and subtropical parts of the world, are one of the most studied plants in the discipline of plant tissue culture and biotechnology. Their huge economic importance, problems associated with conventional propagation (see section 6.1),

the demand for large quantities and good quality planting material and the presence of somaclonal variants in tissue culture systems makes them one of the highly prioritized research crops. After the first notable report on banana tissue culture by Ma and Shii in 1972 (see VUYLSTEKE, 1998 for detail), an upsurge in research on banana tissue culture and related studies was observed from all corners of the world. Research outputs of more than three decades on banana tissue culture are reviewed and documented by VUYLSTEKE (1998). This review will therefore, pay special emphasis only on one of the most limiting factors (problems) of the banana micropropagation industry, somaclonal variation.

2.4.2 Definition and types of somaclonal variation

After the first observation and report by BRAUN (1959), somaclonal variation has been and still is the major problem of many tissue cultured plants, banana being among the most affected (STOVER, 1987; REUVENI and ISRAELI, 1990; BAIRU *et al*., 2006). It is defined as variation originating in cell and tissue cultures (LARKIN and SCOWCROFT, 1981). Currently this definition and/or name is universally adopted or used although there are terms like protoclonal and gametoclonal variation used to describe variants of protoplast and anther culture respectively (KARP, 1994). Somaclonal variation involves all forms of variation in tissue culture. Some scientists add another aspect to the definition and require that somaclonal variation be heritable through a sexual cycle. Unfortunately, it is not always possible or feasible to demonstrate heritability because of complex sexual incompatibilities, seedlessness, and polyploidy or long generation times. Therefore, explaining the heritable nature of somaclonal variation for these types of crops could be difficult or impossible (SKIRVIN *et al*., 1994).

In contrast to spontaneous mutations *in vivo*, *in vitro* generated variations seem to occur more frequently, and are detected more easily since variants can be readily spotted in a limited space and within a short time. This can be verified by considering the occurrence of an albino variant in a Petri dish as compared to that in a field of spaced plants. The exposure of unprotected genetic material to chemicals in the medium and survival of the resulting variants in a non-selective environment increases the mutation rate several fold over that in glasshouse or

field grown plant populations. Even if the rate of mutagenesis is the same in cell and tissue cultures as in field-grown plants, the sheer number of occurrences in a cell population (10^6 after 20 cell divisions) would make accumulation of mutants far greater than in field-grown plants. Hence, somaclonal variants can be detected more frequently in cell cultures than mutations in field grown populations (AHLOOWALIA, 1986).

The *in vitro* culture of plant material can induce or reveal variation between cells, tissues and organs thereby creating variation within cultures, or between the plants derived from them. Some, or all, of the regenerated plants may be physically different from the stock plants from which the culture was derived. Variability of this kind, which usually occurs spontaneously and is largely uncontrolled or directed, can be of two different kinds. These are, firstly, changes caused by cells having undergone persistent genetic change and secondly, those caused by temporary changes to cells or tissues, which is either genetically or environmentally induced (PIERIK, 1987; KARP, 1994).

There are many types of somaclonal variation. These include individuals exhibiting:

- Physical and morphological changes in undifferentiated callus;
- Differences in the ability to organize and form organs *in vitro*;
- Changes manifested among differentiated plants; and
- Chromosomal changes (SKIRVIN *et al*., 1993).

Off-type plants differ from the source plant permanently or temporarily. Temporary changes are an epigenetic or physiological effect and are non-heritable and reversible. The permanent variants referred to, as somaclonal variants are heritable and represents an expression of pre-existing variation in the source plant or are due to the *de novo* variation via an undetermined genetic mechanism (LARKIN and SCOWCROFT, 1981).

2.4.3 Origin and sources of somaclonal variation

Definitively, the causes of somaclonal variation are not always well understood and have not been elucidated. Although studied extensively, the causes remain

largely theoretical or unknown (SKIRVIN *et al.,* 1993; 1994). EVANS and SHARP (1986) stated that among the heritable types of variation, single base-pair changes, chromosome deletion, translocation and changes in ploidy levels have been encountered. Generally variation in tissue culture could either be pre-existing or tissue culture induced (GEORGE, 1993). GENGENBACH and UMBECK (1982) demonstrated that somaclonal variation is not limited to nuclear DNA. By using restriction enzyme analysis of isolated mitochondrial DNA they showed variation in mitochondrially controlled male sterility. This could suggest that variation could occur in the whole plant genome.

i) Pre-existing variation

Heritable cellular variation could result from mutations, epigenetic changes, or a combination of both mechanisms. The distinction between the two mechanisms is an important one, since genetic mutations are essentially irreversible and are likely to persist in the progeny of regenerated plants, whereas epigenetic changes are not transmitted by sexual reproduction (GEORGE, 1993). Use of chimeric plants (GEORGE, 1993; McPHEETERS and SKIRVIN, 1983), variation in ploidy level (BRIGHT *et al*., 1983), tissue culture-induced chromosome aberrations and rearrangement (BRYANT, 1976; LEE and PHILLIPS, 1988), mechanisms regulating the cell cycle (KAPLAN, 1992; BEEMSTER *et al*., 2003), activation of cryptic transposable elements (PESCHKE *et al*., 1987) are some of the factors thought to induce pre-existing variations and are briefly discussed below.

a) Use of chimeras

Chimeras are a source of pre-existing variation *in vitro* (GEORGE, 1993). The arrangement of the genetically different tissues within the plant meristem affects chimera stability. For instance McPHEETERS and SKIRVIN (1983) reported that nearly half of the tissue obtained from tissue culture of a chimeral thornless blackberry were dwarfed and pure thornless. This can best explain the importance of the inherent genetic composition and genome uniformity of the mother plant that is used as starting material for tissue culture. It is more likely that more than one explant is taken from one plant which could cause variation.

Therefore, it is imperative to assess the entire plant for genetic uniformity before using it for tissue culture.

b) Chromosome aberration and rearrangements

Thorough characterization and classification of tissue culture-induced chromosome aberrations have led to a more complete understanding of somaclonal variation. Variation in chromosome number and structure has been observed among cultured cells and regenerated plants. Detailed studies have indicated that structural chromosome changes most accurately reflect the frequency and extent of karyotypic changes. In cultured cells the predominant type of aberration is the result of changes in chromosome structure. Therefore events leading to chromosome breakage, and in some instances subsequent exchange or reunion of fragments, appear to be of fundamental importance (LEE and PHILLIPS, 1988).

BRYANT (1976) indicated that late – replicating heterochromatin and nucleotide pool imbalance are two possible origins of chromosome rearrangement in tissue culture. The former involves the mitotic cell cycle of higher organisms. This cell cycle consists of four phases, G1 (gap), S (synthesis of DNA), G2 (gap), M (mitosis consisting of prophase, metaphase, anaphase, and telophase), each with a species specific and cell type specific duration. Any perturbation affecting the synchrony between chromosome replication during S phase and cell division would likely result in chromosome aberration. Because heterochromatic regions replicate later than euchromatic segments, their integrity may be particularly vulnerable to fluctuations in the cycle (LEE and PHILLIPS, 1988).

c) The role of the cell cycle

The regulatory mechanism of the cell cycle can play a direct role in plant growth and morphogenesis. KAPLAN, (1992) proposed two opposing views; the ‘cell theory’ and the ‘organismal theory’ to address the function of the cell cycle in the growth process. The former considers cells as the building blocks of an organism any increase in cell number causes growth. While the latter considers cell division

as a consequence rather than cause of growth. In their review BEEMSTER *et al*. (2003), however, explained that ‘cells are not autonomous and their growth and development is governed by a variety of signalling and growth substances that are, in turn, synthesised by individual or groups of cells, either within the same or in a different organ’. As a result they proposed an integrated model of the two theories. This suggests that any effect on a cell or organ could possibly induce an effect to the whole organism.

In view of the complicated role of the cell cycle in controlling the growth and morphogenesis of plants, the problems in cell cycle control can possibly create errors during tissue culture that may alter the normal life phenomenon. KARP (1994) explained that during protoplast cultures, for example, a high frequency of errors in microtubule synthesis, spindle formation, spindle orientation, chromatid segregation and cross wall formation occurs which, result in variation in chromosome number and structure. This suggests that any operation that alters the normal process of the cell cycle can cause somaclonal variation.

Evidence supporting the importance of mitotic recombination in generating somaclonal variation is lacking. However, with a few noteworthy exceptions, mitotic recombination including somatic crossing-over and sister-chromatid exchange could produce several types of chromosome rearrangements observed in tissue culture, especially if the exchanges were symmetric or between non-homologous chromosomes (LARKIN and SCOWCROFT, 1981). There are other avenues of chromosome rearrangement and many forms of somaclonal variation. Some examples of somaclonal variation such as morphology of regenerated plants, position effects, qualitative variation and chromosome rearrangements, changes in sequence copy number and gene amplification are involved with chromosome rearrangements (LEE and PHILLIPS, 1988).

d) Activation of cryptic transposable elements

Activation of cryptic transposable elements is another source of chromosome based somaclonal variation. The discovery of activation of maize transposable elements in tissue culture suggested a possible relationship between somaclonal

variation and mobile elements. Chromosome breakage is a means for initiating activity of maize transposable elements (PESCHKE *et al*., 1987). To test for pre-existing somaclonal variation, regenerants may be subjected to another round of *in vitro* regeneration. Clones with pre-existing variation should yield more variability in the first generation than in the second and thereafter variation should be eliminated or stabilized. Subsequent variation is more likely to be tissue culture-derived (SKIRVIN *et al*., 1994).

ii) Tissue culture-induced variation

Although the causes of somaclonal variation are not well understood, it is generally agreed that the method of vegetative propagation used, using chimeral plants, the type of growth regulator, the type of tissue and starting material used, genotype and number and time of sub-cultures are factors determining the chance and frequency of variation during *in vitro* culture (PIERIK, 1987).

a) Methods of vegetative propagation used

Cellular organisation is important in terms of describing origin and cause of somaclonal variation. Tissue culture involves disorganised growth at various levels, from those systems which least disturb the cellular organization such as meristem tip culture to systems such as protoplasts and explant cultures where regeneration is achieved through the formation of adventitious meristems after a phase of disorganised callus or cell suspension culture. Systems subject to instability and disorganised growth suggested that cellular organization is a critical feature and that somaclonal variation is related to disorganised growth (KARP, 1994). Generally, the more the organizational structure of the plant is broken down, the greater the chance of mutations occurring. It is possible to preserve genetic stability to a certain extent by using a single-node or the axiliary-bud method for *in vitro* propagation. If adventitious shoot formation occurs as a result of the use of regulators then the chance of mutations occurring is increased (PIERIK, 1987). Although the direct formation of plant structures from cultured plant tissue, without any intermediate callus phase, minimizes the chance of instability, the stabilizing influence of the meristem is usually lost when plants are grown in culture (KARP, 1994).

b) Types of tissue or starting material used

The use of undifferentiated tissue such as the pericycle, procambium and cambium as starting material for tissue culture reduces the chance of variation. Gross changes in the genome including endo-polyploidy, polyteny and amplification or diminution of DNA sequences could also occur during somatic differentiation in normal plant growth and development (D'AMATO, 1977). Tissue source therefore can affect the frequency and nature of somaclonal variation. The process of de-differentiation and re-differentiation may involve both qualitative and quantitative changes in the genome and different DNA sequences may be amplified or deleted during these changes in the state of the cell that is related to the original tissue source and regeneration system. Somaclonal variation, therefore, can arise from somatic mutations already present in the donor plant (KARP, 1994)

c) *Types and concentration of PGR in use*

In a review, KARP (1994) explained that evidence for direct mutagenic action of growth regulators is somewhat contradictory and most evidence point to a more indirect effect through stimulation of rapid disorganised growth. Growth regulators can also cause transient modifications of phenotypes, although inherited mitotically during plant growth, and not sexually transmitted they are therefore, epigenetic. Inadequate gas exchange in a closed vessel in tissue culture may also result in accumulation of growth regulators such as ethylene which cause epigenetic changes (KARP, 1994).

BAYLISS (1980) reported that plant growth regulators might preferentially increase the rate of division in those cells already genetically abnormal. D'AMATO (1975) also stated that the genetic composition of a cell population can be influenced by the relative levels of both cytokinins and auxins and cells of normal ploidy are often seen to be at a relative advantage in media where these chemicals are present in low concentrations or totally absent. In cultures of unorganised calli or cell suspension, auxin was found to increase genetic variation by increasing the rate of DNA-methylation (LoSCHIAVO *et al*., 1989). The synthetic auxin 2,4-D that is frequently used in callus and cell cultures is often associated with genetic

abnormalities such as polyploidy and the stimulation of DNA synthesis that may result in endoreduplication (SWARTZ, 1991). According to SWARTZ (1991) unbalanced concentrations of auxins and cytokinins are most likely to induce polyploidy. OONO 1982, cited in GEORGE (1993) reported that high levels of BA (30 mg l^{-1}) greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with 2 mg l^{-1} BA.

REUVENI *et al*. (1993) indicated that variation in other medium components used *in vitro* did not directly affect the rate of somaclonal variation in 'Cavendish' bananas. Even with high levels of cytokinins in the medium, which induces the formation of adventitious meristems, the main effect was that of genotype. Despite the recognized importance of hormone levels on shoot regeneration *in vitro* and *ex-vitro*, very few exogenous hormone studies have been reported in banana micropropagation (ZAFFARI *et al*., 2000). Sub- and supra-optimal levels of plant growth substances in banana tissue culture media have been associated with somaclonal variation (STOVER, 1987). However, REUVENI and ISRAELI (1990) studied the effect of growth regulators and rate of multiplication and found that these do not affect somaclonal variation in banana. SCOWCROFT (1984) also found that culture media and growth regulators appear not to be mutagenic.

d) Number and time of sub-culturing

SWARTZ (1991) stated that increasing the number of sub-cultures and their duration favours the rate of somaclonal variation. Somaclonal variation is high when a high rate of proliferation is achieved, the period between sub-cultures is short and more sub-cultures are performed in a given time (ISRAELI *et al*., 1995). REUVENI and ISRAELI (1988) stated that with a relatively constant period of time between sub-cultures, the rate of somaclonal variation increases with generation number. RODRIGUES *et al*. (1998) also showed that somaclonal variants appeared from the fifth sub-culture (1.3%) onwards and increased to 3.8% after 11 sub-cultures. This variation in culture also differs among cultivars. HWANG and KO (1987) for instance reported an overall variation rate of 3% among banana cultures, but the rate of variation for 'Cavendish' banana went as high as 20%, a rather disconcerting development.

In a review LEE and PHILLIPS (1988) explained that plant cells in tissue culture might be especially susceptible to nucleotide pool imbalances (dNTP) because they can be serially transferred from depleted to fresh media almost indefinitely. Intracellular deoxyribonucleotide pools have an important influence on the fidelity of several components of prokaryotic and eukaryotic DNA metabolism; including precursor biosynthesis, replication, DNA repair, recombination, and possibly degradation. Imbalances in the dNTP pools may have serious consequences such as nuclear chloroplast and mitochondrial mutation, mitotic recombination, chromosome (structural) aberrations, aneuploidy, sister chromatid exchange, increased sensitivity to mutagens and oncogenic transformation.

e) Effect of stress and genotype

A stress during tissue culture can also induce somaclonal variation. Different genomes however, respond differently to this stress-caused variation indicating that somaclonal variation has genotypic components. The differences in stability are related to differences in genetic make-up whereby some components of the plant genome make them unstable during the culture process. This could be better explained by the repetitive DNA sequences, which can differ in quality and quantity between plant species (LEE and PHILLIPS, 1988).

DAMASCO *et al*. (1998a) reported that the inherent instability of a cultivar is another major factor that influences dwarf off-type production in banana tissue culture. They found that cv. ‘New Guinea Cavendish’ has a higher level of instability *in vitro* than cv. ‘Williams’. They further demonstrated that the dwarf off-types were stable *in vitro*, and the tissue culture conditions that induced dwarfism in normal plants did not induce reversion of the dwarf off-type trait. Multiplication of the already existing dwarf off-types on the subsequent sub-cultures, along with the irreversible nature of the dwarf off-types *in vitro,* plays a significant role in raising the percentage of the dwarf population. This situation stimulates interest to study factors governing or influencing genetic stability *in vitro*.

ETIENNE and BERTRAND (2003) reported that embryogenic cell suspension age and genotype affected the frequency and phenotype of variants produced

significantly in *Coffea arabica*. The severity of variation also increased with cell suspension age. MEHTA and ANGRA (2000) indicated that there was variation in disease resistance among regenerants of somaclones of wheat cultivars. In another report POPESCU *et al*. (1997) demonstrated that both genotype and type of explant strongly influenced occurrence of somaclonal variation in callus cultures of strawberry.

2.4.4 Description and occurrence of variants

Dwarfism, accounting for 75% (STOVER, 1987) to 80% (REUVENI and ISRAELI, 1990) of the total variants, is the most common variation observed in the 'Cavendish' banana sub-group. Variation in stature is a means of characterization for various variants. For example in 'Williams' and 'Grand Naine' the off-types are very similar to 'Dwarf Cavendish', while in the Israeli selection 'Nathan' (derived from 'Dwarf Cavendish') the most common variant is an extra-dwarf type (ISRAELI *et al*., 1991) which, is an unwanted quality in terms of uniformity of production and yield parameters.

Variation in foliage is another common type of variation, the mosaic type being the most common (REUVENI and ISRAELI, 1990). Thick, rubbery, narrow leaves characterize it, with different degrees of pale-green mottling resembling a virus infection to the inexperienced eye (ISRAELI *et al*., 1991). There is also variation in colour and morphology of pseudostems such as height, circumference and spacing between petioles. DANIELLS and SMITH (1991) reported an extremely thin pseudostem. ISRAELI *et al*. (1991) and DANIELLS and SMITH (1991) also indicated changes in colour involving various degrees of black, reddish, pale-green and brown associated with colour changes of petioles and midribs. Variation in reproductive organs was also reported. Nipple-like tips, bunches with only male flowers (STOVER, 1987), persistent flowers, split fingers (ISRAELI *et al*., 1991), changes in bract colour, and in the shape and colour of male buds (STOVER, 1987; DANIELLS and SMITH, 1991) were noticed. There are also unexposed variants some of which are detected for their usefulness. SMITH and DREW (1990b) for example, indicated variants of 'Mons Mari' having extra-long fingers and a dwarf type with no obvious choke-throat problems. HWANG and KO (1987)

also screened and selected a somaclonal variant resistant to race 4 of *Fusarium* wilt.

2.4.5 Detection and/or characterization of variants

Present commercial procedures reduce the proportion of off-type plants that reach the farmer to the commercially acceptable rate, below 5%, (HWANG and KO, 1987) even though percentages as high as 29% (REUVENI and ISRAELI, 1988), 80% (RODRIGUES *et al*., 1998) and 90% (SMITH and DREW, 1990b) were reported. Early detection and elimination of off-types or reducing the rate to below the commercially acceptable rate during the process of *in vitro* production is crucial. Optimising *in vitro* techniques (REUVENI and ISRAELI, 1990), morphological parameters for selecting off-types during the hardening stage, early detection of off-types with molecular markers (GRILLO *et al.,* 1998) and physiological characterization (DAMASCO *et al.,* 1997) were proposed as means to detect and reduce the rate of variants.

In addition to its use in commercial propagation of tissue cultured plants to remove or minimize off-types before field establishment, understanding the cause and origin of somaclonal variation is important for its realistic potential application to crop impovement.

i) Morphological detection

In field conditions it is possible to detect dwarf off-types by observing the plant stature and leaf index (leaf length/width) 3 to 4 months after establishment (ISRAELI *et al.,* 1991). However, SMITH and DREW (1990b) indicated that sometimes dwarf off-types could not be detected until the flowering and fruiting stage. Detection at this stage is very costly and too late to replace variants. Therefore, it becomes very important to detect off-types at earlier stages *in vitro* and in the nursery. Dwarf off-types, for example, can be detected by measuring differences in plant height, petiole length and leaf morphology carefully in the nursery (DANIELLS and SMITH, 1991; ISRAELI *et al.,* 1991). ISRAELI *et al.* (1991) indicated that in the nursery dwarf off-types are approximately 5 cm and 10 cm shorter than the normal plant for ‘Grand Naine’ and ‘Williams’ respectively.

Though it is not convenient for routine detection, SANDOVAL (1994) reported that detection of dwarf and giant off-types of 'Grand Naine' was possible using a combination of leaf characteristics *in vitro*.

ii) Physiological characterization

DAMASCO *et al.* (1996a) demonstrated the application of gibberellic acid (GA_3) and photo-inhibition responses to detect somaclonal variants. The former involves an exogenous application of GA_3 at the *in vitro* or acclimatization stages to enhance morphological differences between dwarf off-types and normal plants. 'Cavendish' dwarf off-types are considered to be GA_3 non-responsive (REUVENI, 1990). SANDOVAL *et al.* (1995) also demonstrated that dwarf off-types have lower levels of endogenous gibberellins compared to normal plants. For effective utilization of this technique it is necessary to determine the GA_3 concentration and timing of application that gives maximum discrimination between normal and dwarf off-types (DAMASCO *et al.,* 1998b).

DAMASCO *et al.* (1997) indicated that leaf yellowing, due to photo-oxidation of chlorophyll, was more severe in normal plants than in dwarf off-types in the field. The decrease in chlorophyll fluorescence ratio F_v/F_m was also significantly greater in normal plants than dwarf off-types. 'Cavendish' banana leaves are susceptible to photoinhibition under controlled experimental conditions. In both attached and detached leaves, the decrease in F_v/F_m was significantly higher in normal plants than in dwarf off-types (DAMASCO *et al.,* 1997).

iii) Molecular detection

a) Proteins and isozymes

Proteins are the most abundant organic molecules found in cells and have many different functions. The most abundant class of proteins, and those used for electrophoretic analysis, are the enzymes, which catalyse metabolic reactions. These enzymes are highly specific both in the reaction they catalyse and the substrates that they modify (ERASMUS, 2001).

This technique involves analysing clones for protein and enzyme polymorphism (JARRET and LITZ, 1986). It detects naturally occurring genetic variability that had arisen for many enzyme proteins, which can be used to type, or fingerprint, individuals with respect to the isozyme variants present (JARRET and LITZ, 1986; ERASMUS, 2001). Subjecting tissue extracts to various types of gel electrophoresis and subsequently incubating in solutions containing enzyme-specific stains reveals isozymes. A good example of the use of isozyme based characterization of *Musa* clones, as mentioned by JARRET and LIZT (1986), is a resolution for heterozygosity obtained after electrophoresis for isozymes of malate dehydrogenase (MDH), phosphoglucomutase (PGM), glutamate oxaloacetate transmaninase (GOT), shikimate dehydrogenase (SKDH) and peroxidase (PRX). Polymorphism was detected in all 5 enzyme systems.

According to JERRET and GAWEL (1995) total protein and isozyme analysis has also been used in *Musa* for species and cultivar differentiation. But this technique does not reveal differences between normal and dwarf plants and mosaic-like variants in 'Williams'. It has also very limited potential in detecting somaclonal variants in *Musa*. Since isozyme markers represent only a small portion of the genome, there is a very low chance that a mutation event would involve a particular enzyme-coding gene. They are unstable markers (DAMASCO *et al.,* 1998b).

b) Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another (BROWN, 1995).

Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme

recognition sites are usually 4 to 6 base pairs in length. Generally, the shorter the recognition sequence, the greater the number of fragments generated. If molecules differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be part of the cell's defenses against invading bacteria and viruses. These enzymes are named by using the first letter of the genus, the first two letters of the species, and the order of discovery (BROWN, 1995).

RFLP analysis has been used in *Musa* species to detect genetic diversity and to differentiate between A and B genomes (JARRET and GAWELL, 1995). Characterization of *Musa* somaclonal variants using this method has not been undertaken due to the following technical limitations (KARP *et al*., 1996; DAMASCO *et al*., 1998b):

- A good supply of probes is needed and, if heterologous probes are unavailable, cDNA or genomic DNA probes must be developed;
- The blotting and hybridisation steps are time consuming and difficult to automate; and
- Sufficient quantities of good quality DNA (up to 10 μg per digestion) are required. RFLPs are, thus, not applicable where very limited amounts of source material (such *as in vitro* plants) or preserved tissues are available.

c) The polymerase chain reaction (PCR)

The development of the PCR for amplifying DNA brought an advancement in the applicability of molecular methods and a range of new technologies were developed which can overcome many of the technical limitations of RFLPs. PCR is a technique for amplifying a specific region of DNA, defined by a set of two short oligonucleotides at which DNA synthesis is initiated by a thermo-stable DNA polymerase. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realized and the PCR product can be detected by gel electrophoresis. The most common version of PCR is RAPD (random amplified polymorphic DNA) analysis, in which the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet

light. The regions amplified are usually between 150-3,000 base pairs in length (BROWN, 1995; KARP *et al*., 1996).

Polymerase Chain Reaction (PCR)-based techniques offer much potential for detecting somaclonal variants in micropropagated bananas. The advantages of PCR – based genetic markers over other markers such as allozymes and RFLPs include: (MORELL *et al*., 1995; KARP *et al*., 1996; DAMASCO *et al*., 1998b)

- The PCR assay is easy to perform and requires small amounts of DNA (as small as 10 ng);
- For RAPD (random amplified polymorphic DNA), no prior sequence information is required since a number of commercially available arbitrary primers can be used;
- The technique is convenient to use for screening large numbers of plants;
- Since the procedure involves no blotting or hybridising steps, it is quick, simple and automatable. It is absolutely critical, however, to maintain strictly constant PCR reaction conditions in order to achieve reproducible profiles;
- PCR involves fewer steps compared to RFLPs and it is technically straight forward;
- PCR does not require the use of radioactivity;
- At most stages PCR is automated from DNA extraction to data collection and analysis; and
- The vast range of potential primer sequences gives the PCR great diagnostic power.

HOWELL *et al.* (1994) reported that RAPD analysis did not reveal variation between normal and off-type plants from micropropagated ‘Cavendish’ banana cultivar ‘Valery’. DAMASCO *et al*. (1996b), however, found a RAPD marker using primer OPJ-04, 5’-CCGAACACGG-3’ specific to ‘Cavendish’ dwarf off-types. GRAJAL-MARTIN *et al*., (1998) also reported that the primers OPC-15 (5’-GACGGATCAG-3’) and OPJ-04 revealed variability in *in vitro* plants.

Due to its cost effectiveness and suitability for small projects (RAGOT and HOISINGTON, 1993), RAPD has been used for a number of studies in the

assessment of genetic variability, determining varietal purity and assessing germplasm (WEEDEN *et al*., 1992). Identification and mapping of polymorphism in wheat (WEINING and LANGRIDGE, 1991), somaclonal variation of pear and apple (CABONI *et al*., 2000), *Populus deltoids* (RANI *et al*., 1995), strawberry (POPESCU *et al*., 1997), coffee (ETIENNE and BERTRAND, 2003), maize (EDWARDS, 2000), turfgrass breeding (PEAKALL, 1997), study of clonality in *Haloragodendron lucasii* (SYDES and PEAKALL, 1998) and disease resistance in wheat (MEHTA and ANGRA, 2000) are among the few examples that utilized RAPD analysis. For more details about PCR and its technical requirements refer to Appendix 2 c.

2.5 Concluding remarks

The reviews discussed in four sections above intend to provide some scientific background and the required knowledge to understand and explain the results of the following experimental chapters in addition to identifying the gap in knowledge. Due to the diverse nature of the problems investigated, at most effort was made to highlight only the relevant literatures and limit the scope of the review within the range of the topics. The common elements of the chapters, the cytokinins, are discussed in relation to the micropropagation problems studied and the basic knowledge of cytokinins including their biosynthesis, metabolism and activity. The complex nature of problems related to micropropagation made it imperative to cover as many interrelated topics as possible to try and understand the mechanisms by which each problem affects plant growth and development. It is therefore, important to highlight that the experimental chapters of this thesis do not cover each and every topic in the review. But the contents of the review are used in one way or another, to at least explain some of the results. Factors affecting cytokinin activity, hyperhydricity, STN and somaclonal variation are emphasised.

3. Comparative study of the biological activity of selected cytokinins using the soybean callus bioassay

3.1 Introduction

The past half a century witnessed a tremendous upsurge in research in structure-activity relationship studies of small organic molecules in a wide range of disciplines including the study of plant growth regulators (CORSE *et al*., 1989), Included cytokinins (LEONARD *et al*., 1969; HECHT *et al*., 1975; IWAMURA *et al*., 1980; MATSUBARA, 1980; KAMINEK *et al*., 1987a; HOLUB *et al*., 1998; and many more). It however, remains important to compare the biological activity of particular cytokinins and their derivatives before embarking to more applied research such as plant tissue cultures. Information from the study of structure-activity relationships helps to elucidate the mechanism of cytokinin action and provides clues on the receptor sites involved in the various physiological processes (CORSE *et al*., 1989).

The discovery of aromatic cytokinins as naturally occurring compounds (HORGAN et at., 1975) has revolutionized cytokinin research both with respect to the chemistry and application in tissue culture of these compounds. There is now growing interest to research the potential of aromatic cytokinins. The presence of a wide range of natural analogues of these aromatic cytokinins with potent activity has made the search and research more interesting. Hydroxybenzyladenines, now commonly called topolins (STRNAD *et al*., 1997), are members of these interesting classes of compounds. Research on topolins and their derivatives is based on the working hypothesis set by STRNAD *et al*. (1992) which states that ‘hydroxybenzyladenines and other related plant-derived compounds with some biological activity are natural analogues of the cytokinin BA. Further research is now focused on identification, synthesis and activity analysis of these analogues (TARKOWSKÁ *et al*., 2003; DOLEŽAL *et al*., 2006; DOLEŽAL *et al*., 2007a).

It has long been known that cytokinin activity depends on the side chain configuration. For example HECHT *et al*. (1975) attributed the higher activity of *trans*-zeatin compared to its *cis* isomer to the co-planarity of the side chain. Differential influence of the position of the hydroxyl group on the N^6 side-chain was also reported with the 3,4-hydroxy derivatives being more active than their 2,3-hydroxy counterparts (LEONARD *et al*., 1969). Slight structural differences within the topolins affected the biological activity of these compounds (HOLUB *et al*., 1998). Major differences in cytokinin activity of different topolin isomers can be explained by differences in their molecular structures. (TRÁVNÍČEK *et al*., 1997), The presence of the hydrogen bond between the N(1) atom and OH group of the benzyl ring entails a considerable decrease in the dihedral angle between the benzyl ring and purine skeleton of the *ortho*-topolin molecule. This dihedral angle is equal to 62.7°. Similar deformation (65.3°) was recently found for the inactive *para*-derivative due to the presence of several intermolecular hydrogen bonds. In contrast, the same angle in cytokinin active BAPR is 98.9° (KORSZUN *et al*., 1989). It is also clear, that similar hydrogen bonds can not exist in case of methoxy derivatives, because of the lack of free OH groups in their molecules.

Structural modification with the addition of halogens also influences cytokinin activity. It is now possible to synthesize these analogues by replacing specific protons by halogens such as chlorine and fluorine. Apart from their use to study plant physiology and biochemistry and use as commercial growth regulators, these halogenated derivatives are playing an important role in the study of some structural requirements and biological activity of plant growth regulators (REINECKE and OZGA, 1999). Fluorinated iP and zeatin are more active than their corresponding free bases. Two derivatives of N-6-isopentenyladenine, bearing either a vinylic fluorine atom or a trifluoromethyl group, were synthesized from known fluorinated precursors (CLEMENCEAU *et al*., 1996). Both derivatives were found to be more active than the parent compounds, zeatin and N-6-isopentenyladenine. Two such compounds are included in this study for comparison (Figures 3.1 M and O).

Cytokinin structure-activity relationships are well researched. The biological activity of cytokinins varies considerably with change in structure, which in most

cases is the result of the modification of the side chain. It should however, be noted that the activity of cytokinins could vary from bioassay to bioassay depending on the type of biological response examined and the plant material selected for the study. Differences in cytokinin uptake, metabolism and difference in structural requirements for activity at the site of action could be possible causes for a difference in structure activity relationships (MOK *et al*., 1978). Hydroxylation of the benzyl ring on the *meta* position increased the activity in tobacco callus and chlorophyll retention bioassays compared to *ortho*-hydroxylation but decreased activity in the *Amaranthus* bioassay (HOLUB *et al*., 1998). A general trend in biological activity of aromatic substituents is in the order of *meta* > *para* > *ortho* for both phenyl- and diphenyl-urea derivatives (IWAMURA *et al*., 1980). Conversely, KAMÍNEK *et al*. (1987a) reported that the activity of hydroxylated derivatives of BA is in the order of free base > *meta* > *ortho* > *para*. Care should therefore, be taken during interpreting and analysing activity results of the various bioassay systems. It should also be noted that there may not always be a direct correlation between activity in bioassay systems and effects on morphogenesis or lateral bud growth of a given cytokinin (KAMÍNEK *et al*., 1987a and b).

3.2 Materials and methods

All the cytokinins (except BA, kinetin and zeatin) were prepared as published previously (TARKOWSKÁ *et al*., 2003; DOLEŽAL *et al*., 2006; DOLEŽAL *et al*., 2007a and b; SZÜČOVÁ *et al*., 2007). BA, kinetin and zeatin were purchased from SIGMA. The soybean callus bioassay developed by MILLER (1965), as used by VAN STADEN and DREWES (1991), was employed to determine the biological activity of the different cytokinins. Seventeen cytokinins (Table 3.1 and Figure 3.2) were each used in a range of concentrations (0.001, 0.01, 0.1, 1, 10 and 100 µM serial dilution) in 15 ml Miller's media solidified with 1% agar (Oxoid No. 1). The control contained Miller's media with no added cytokinin. There were four replicates per treatment. Three pieces of soybean callus (approximately 10 mg) were then transferred to each flask aseptically. Cultures were maintained for 28 days at 25±2°C under a low light intensity of 0.5 µmol m^{-2} s^{-1}. Data were collected as fresh mass of callus per flask and subjected to analysis of variance (SPSS version 13.0). Activity was expressed as mean mass of callus per treatment per

concentration and significance test at 5% probability level. Results were then analyzed from two perspectives; considering the different types of cytokinins as sources of variation (Table 3.2) and the different concentrations as sources of variation (Figure 3.1).

3.3 Results and discussion

The results of the soybean callus bioassay are presented in Table 3.2 and Figure 3.1 representing differences between the various cytokinins and between the concentrations respectively. The cytokinins tested varied considerably in their activity. Of all the topolins tested *p*T, its methoxy (Me*p*T) and methoxy riboside (Me*p*TR) derivatives and *o*T showed the least activity. This is in agreement with previous reports (MATSUBARA, 1980; KAMINEK *et al*., 1987a). Neither the methoxy nor the methoxy riboside forms of this compound improved activity. The effect of a terminal hydroxy group and position specificity in cytokinin activity has long been understood. Significant effects on the activity of cytokinins have been observed by altering the position of the hydroxyl group on the side chain (KAMINEK *et al*., 1979; IWAMURA *et al*., 1980).

The methoxy (Me*o*T) and methoxy riboside (Me*o*TR) forms of *o*T significantly improved activity compared to *o*T. These derivatives of *o*T showed the highest activity at a concentration of 10 µM. This significant increase in activity can be explained in two ways. First *o*T forms a hydrogen (H) bond between the OH group on the benzyl ring and the nitrogen at the N-1 position. This H bond is thought to be responsible for the intermolecular inactivation. The addition of the methoxy group prevents the formation of this bond, thereby increasing its activity. Secondly, the H bond between the N-1 and H-16 atoms imposes a considerable decrease in the dihedral angle between least-square planes fitted through the purine and phenyl ring. This reduction in dihedral angle reduces activity (TRAVNICEK *et al*., 1997). It is also clear that the presence of the methyl group in the molecular structures of methoxytopolins prevents the formation of the H bond thereby maintaining the angle. In addition, MATSUBARA (1980) mentioned that removing the methyl group from *cis*-zeatin reduced its activity more than five-fold. This is an

indication that the methyl group plays a major role in increasing the activity of Me*o*T and Me*o*TR. The methyl group is very important in determining cytokinin activity, with activity being highly affected by the position of the methyl group (MATSUBARA, 1980). The shift of the methyl group from the 3- to 2- position in *cis*- and *trans*-zeatin resulted in deceased activity, of up to 100 times, and removing it completely from *cis*-zeatin resulted in significant loss of activity (SCHIMTZ *et al* 1972). The high activity of this compound in the soybean callus bioassay could therefore, be attributed to the methyl group.

Results of *m*T and derivatives also showed a similar trend with exception of Me*m*T which had slightly lower activity (Figure 3.1B - E). The activity trend was Me*m*TR > *m*TR > *m*T > Me*m*T. This result and results from the *p*T and derivatives show that the methyl group *per se* is not responsible for the overall increase or decrease in activity. It is also important to note the relatively higher activity observed by these compounds at the highest concentration whereas BA was toxic at this concentration (Figure 3.1 A - E). This indicates that *m*T and derivatives are less toxic than BA at higher concentration. This is in agreement with the literature. It was suggested by DOLEŽAL *et al*. (2006) that this BA toxicity is at least partially caused by inhibition of endogenous cycline dependent kinase (CDK) -like kinases which play regulatory roles in cell division. Similar results are found in tissue culture systems (see chapter 4). These results could have great practical significance should there be a need to use higher hormone doses. Such use has been reported in an *ex vitro* experiments on shoot and lateral bud induction in *Poinsettia* and gerbera daisy (KAMINEK *et al*., 1987b).

KAMINEK *et al*., (1979) demonstrated that the hydroxylation of the *trans* methyl group in the N^6 side chain of N^6-(Δ^2-isopentenyl) adenosine increased the biological activity. This activity either decreased or was not significantly affected when the *cis* methyl group was hydroxylated in four bioassays namely, stimulation of cucumber cotyledon expansion, retention of chlorophyll in detached leaf pieces, induction and stimulation of chlorophyll synthesis in cucumber cotyledons and betacyanin synthesis in *Amaranthus caudatus* seedlings. The soybean callus bioassay employed in this study showed that hydroxylation of BA at the *ortho* and *para* positions (as in *o*T and *p*T) decreased activity. However, activity increased

when the hydroxylation was on the *meta* position (as in *m*T) (Table 3.2 and Figure 3.1A-K). This result is similar to that of KAMINEK *et al*., (1987a). They tested cytokinin activity of BA and its derivatives hydroxylated on the side chain phenyl ring at the *ortho*, *meta* and *para* positions in four bioassays namely, stimulation of growth of tobacco callus, retention of chlorophyll in excised wheat leaves, dark induction of betacyanin synthesis in *Amaranthus* cotyledons and release of lateral buds of pea from apical dominance and found that activity was decreased in the *ortho* and *para* positions but increased in the *meta* position.

Of particular interest is the high biological activity recorded with the derivatives of BA, with BA_9THP having the highest activity at 1 µM (Figures 3.1 L - N). ZHANG and LETHAM (1989) tested the senescence retarding activity of 9-substituted derivatives of BA and found that $BA_9$9THP (Figure 3.1L) and BA_9THF (a compound not included in this assay) were considerably more effective than BA in retarding soybean leaf senescence. These authors attributed their results to the ability of these derivatives to release free BA and their ability to be debenzylated to 9THP-adenine and 9THF-adenine respectively, unlike the weakly active 9-alanine conjugate which was not metabolized in leaf discs. In addition these active derivatives had greater stability and the BA they released was less susceptible to inactivation by alanine conjugate formation as opposed to exogenously applied BA (ZHANG and LETHAM, 1989). These findings could therefore, be logical explanations when interpreting the results of this study. The toxic effect observed at 100 µM by BA9THP could be the result of continuous release of BA by this stable derivative.

3FBA also showed good activity at 1 µM unlike most of the cytokinins tested where highest activity was recorded at 10 or 100 µM. The effect of fluorine substitution on the activity of zeatin analogues had been studied (HAIDOUNE *et al*., 1998 and references therein). The fluorine atom improved the activity of the fluoro-analogue of *cis*-zeatin but had no effect on the fluoro-analogue of zeatin itself (CLEMENCEAU *et al*., 1996; HAIDOUNE *et al*., 1998). These authors suggested that the presence of a vinylic fluorine atom might have made these molecules less sensitive to oxidative cleavage of the aliphatic chain by cytokinin oxidases. They however, indicated that this assumption may not explain the situation with the

fluoro-analogues of zeatin. Instead, they attributed the change in response to the existence of an internal hydrogen bond between the fluorine and the OH hydrogen atoms since they observed no loss of side-chain planarity, which is essential to cytokinin activity (HECHT *et al*., 1970a). High electronegativity and small size are among the special properties in fluorine chemistry (CROCKER *et al*., 2007). The ability of organo-halogen compounds to form structural motifs via inter-molecular interactions is well known. Fluorine makes higher intermolecular interaction than the other halogens (EMMERLING *et al*., 2007). The higher activity observed in 3FBA could, therefore, be due to the better interacting ability of the fluorine atom.

$2Cl_6$(3OHBA)R showed less activity compared to the BA derivatives (Figures 3.1 L-N) although it was slightly more active than both kinetin and zeatin at 10 µM (Table 3.2). The substitution with electron-withdrawing groups can increase cytokinin activity (RICCI *et al*., 2005 and references therein). 6-(3-chloro-trans-2-butenylamino)purine was more active than the *cis* isomer possibly due to the improved ability of the chloro-group to maintain side-chain planarity compared to the *cis* isomer (HECHT *et al*., 1970a). It has also been suggested that the absolute configuration around the asymmetric carbon may contribute to the interaction between the cytokinin molecule and its receptor site (MATSUBARA, 1980) thereby affecting the activity.

HECHT *et al*. (1970b) studied the effect of substitution at position 2 of the purine ring. They found that the chloro-derivatives were consistently more active than their unsubstituted counterparts. The activity of 2-chloro-zeatin was greater or equal to zeatin itself. The results from the present study are contrary to these findings since the chlorine substitution at position 2 reduced the activity compared to that of the free base (BA). One positive observation about this compound however, was the absence of a toxic effect at the highest concentration tested (100 µM), unlike its free base and the other BA derivatives (Figure 3.1 A and L-O).

Table 3.1: Details of the cytokinins tested in the soybean callus bioassay

Name	Symbol	Chemical name	Molecular weight	Type
N^6 -Benzyladenine	BA	[6 – benzylaminopurine]	225.25	Aromatic
Kinetin	K	[6 – furfurylaminopurine]	215.22	Aromatic
meta-Topolin	*m*T	[6-(3-hydroxybenzylamino)purine]	241.25	Aromatic
meta-Topolin riboside	*m*TR	[6-(3-hydroxybenzylamino)-9-β-D-ribofuranosylpurine]	373.37	Aromatic
ortho-Topolin	*o*T	[6-(2-hydroxybenzylamino)purine]	241.25	Aromatic
para-Topolin	*p*T	[6-(4-hydroxybenzylamino)purine]	241.25	Aromatic
meta-Methoxytopolin	Me*m*T	[6-(3-methoxybenzylamino)purine]	255.28	Aromatic
ortho-Methoxytopolin	Me*o*T	[6-(2-methoxybenzylamino)purine]	255.28	Aromatic
para-Methoxytopolin	Me*p*T	[6-(4-methoxybenzylamino)purine]	255.28	Aromatic
meta-Methoxytopolin riboside	Me*m*TR	[6-(3-methoxybenzylamino) -9-β-D-ribofuranosylpurine]	387.40	Aromatic
ortho-Methoxytopolin riboside	Me*o*TR	[6-(2-methoxybenzylamino) -9-β-D-ribofuranosylpurine]	387.40	Aromatic
para-Methoxytopolin riboside	Me*p*TR	[6-(4-methoxybenzylamino) -9-β-D-ribofuranosylpurine]	387.40	Aromatic
Fluorobenzylamino purine	3FBA	6-(3-fluorobenzylamino)purine	243.25	Aromatic
N/A	2Cl6(3OHBA)R	2-chloro-6-(3-hydroxybenzylamono) purine-9-roboside	407.82	Aromatic
N/A	BA_9THP	6-benzylamino-9-tetrahydropyranylpurine	309.37	Aromatic
trans-Zeatin	Z	[6-((E)-4-hydroxy-3-mehtylbut-2-enylamino)purine]	219.25	Isoprenoid

Table 3.2: Comparison of the biological activity of different cytokinins using the soybean callus bioassay

Cytokinins tested	Mean mass of callus (g) at various concentrations (µM)						
	0	**0.001**	**0.01**	**0.1**	**1**	**10**	**100**
BA	0.06±0.03^{a}	0.14±0.07^{bcd}	0.22±0.05^{bcd}	0.41±0.01^{de}	2.41±0.09^{a}	2.66±0.29^{abc}	0.65±0.30^{cd}
*m*T	0.06±0.03^{a}	0.28±0.21^{abc}	0.32±0.06^{b}	0.37±0.09^{de}	1.06±0.26^{cdef}	1.91±0.45^{abcde}	1.77±0.32^{abc}
*m*TR	0.06±0.03^{a}	0.39±0.06^{a}	0.14±0.05^{bcd}	0.34±0.10^{de}	2.08±0.22^{ab}	2.63±0.39^{abc}	2.12±0.37^{abc}
Me*m*T	0.06±0.03^{a}	0.06±0.02^{cd}	0.08±0.03^{cd}	0.53±0.30^{cde}	0.85±0.30^{defg}	1.70±0.64^{bcdef}	2.59±0.12^{ab}
Me*m*TR	0.06±0.03^{a}	0.42±0.08^{a}	0.67±0.14^{a}	0.98±0.05^{abc}	1.30±0.49^{bcde}	2.95±0.24^{ab}	2.44±0.52^{ab}
*o*T	0.06±0.03^{a}	0.03±0.01^{cd}	0.07±0.02^{d}	0.37±0.06^{de}	0.57±0.11^{efg}	1.21±0.57^{def}	0.86±0.31^{cd}
*p*T	0.06±0.03^{a}	0.05±0.02^{cd}	0.04±0.01^{d}	0.02±0.004^{e}	0.06±0.02^{g}	0.51±0.23^{f}	1.77±0.13^{abc}
Me*o*T	0.06±0.03^{a}	0.11±0.07^{cd}	0.025±0.03^{bcd}	0.33±0.16^{de}	1.70±0.44^{abcd}	3.15±0.16^{a}	2.97±0.47^{a}
Me*p*T	0.06±0.03^{a}	0.02±0.003^{d}	0.03±0.008^{d}	0.15±0.07^{e}	0.29±0.05^{fg}	0.53±0.08^{f}	0.65±0.22^{cd}
Me*o*TR	0.06±0.03^{a}	0.04±0.002^{cd}	.031±0.04^{bc}	1.04±0.04^{abc}	0.91±0.09^{defg}	3.02±0.06^{a}	1.98±0.57^{abc}
Me*p*TR	0.06±0.03^{a}	0.05±.0.01^{cd}	0.03±0.002^{d}	0.04±0.008^{e}	0.04±0.006^{g}	0.65±0.10^{ef}	0.04±0.01^{d}
BA_9THP	0.06±0.03^{a}	0.03±0.003^{d}	0.72±0.15^{a}	0.77±0.13^{bcd}	2.18±0.09^{ab}	2.01±0.61^{abcd}	0.02±0.003^{d}
3FBA	0.06±0.03^{a}	0.04±0.01^{cd}	0.34±0.13^{b}	1.16±0.31^{ab}	1.97±0.65^{abc}	1.32±0.69^{def}	0.71±0.10^{cd}
2Cl6(3OHBA)R	0.06±0.03^{a}	0.03±0.008^{cd}	0.04±0.005^{d}	0.26±.07de	0.61±0.25^{efg}	1.74±0.16^{bcdef}	1.99±0.91^{abc}
Kinetin	0.06±0.03^{a}	0.04±0.02^{cd}	0.02±0.003^{d}	.0.08±0.04^{e}	0.45±0.20^{efg}	1.62±0.20^{cdef}	1.45±0.63^{bcd}
Zeatin	0.06±0.03^{a}	0.22±0.03^{abcd}	0.70±0.04^{a}	0.82±0.07^{abcd}	0.91±0.40^{defg}	1.65±0.44^{cdef}	1.21±0.06^{bcd}

Means denoted by the same letters have no statistically significant difference. Please note that this table is made to give readers a closer look at the figures and make comparison between the cytokinins at a given concentration (columns) and/or across the concentrations (rows) and compliments Figure 3.1.

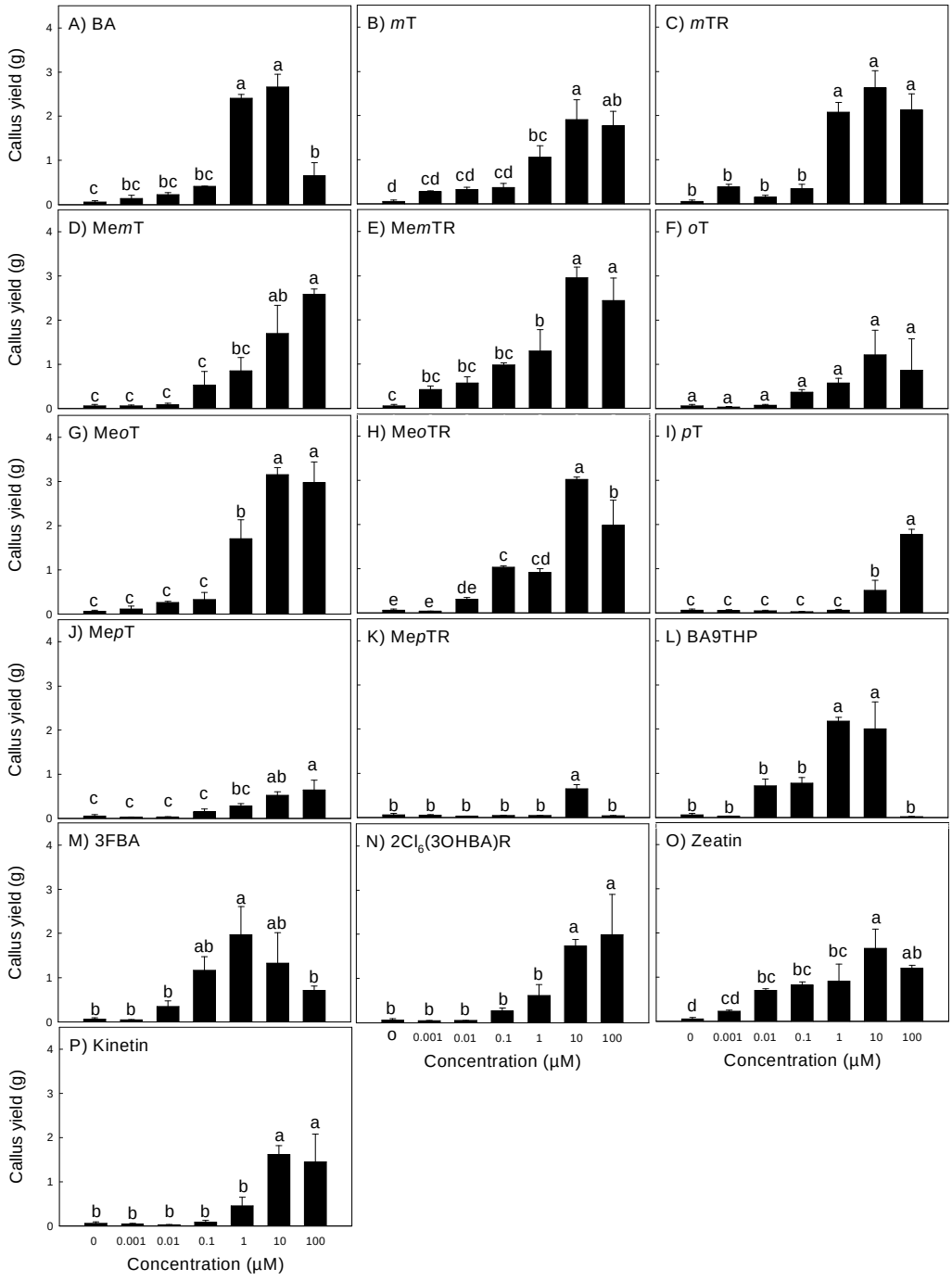
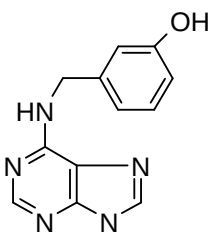
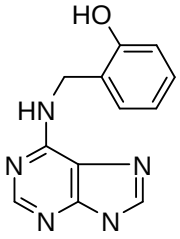


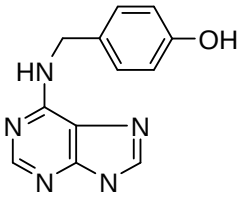
Figure 3.1: Relative biological activities of cytokinins in the soybean callus bioassay (mean callus yield in grams). Means denoted by the same letters are not significantly different statistically.



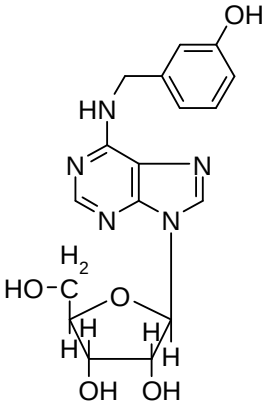
***m*T**



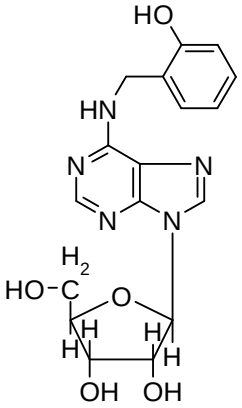
***o*T**



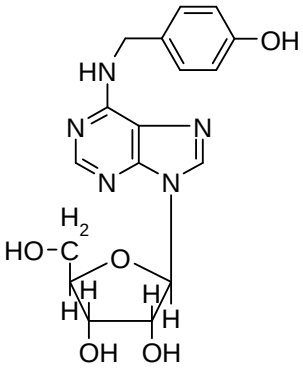
***p*T**



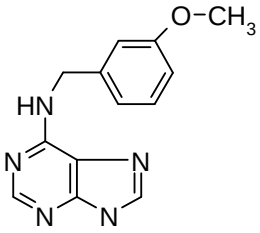
***m*TR**



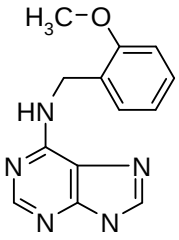
***o*TR**



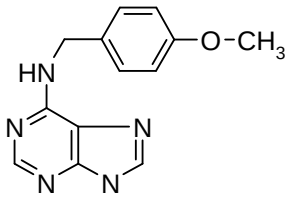
***p*TR**



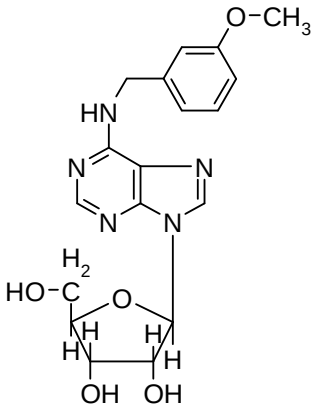
Me*m*T



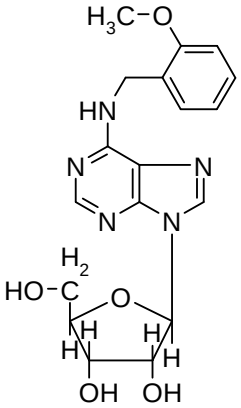
Me*o*T



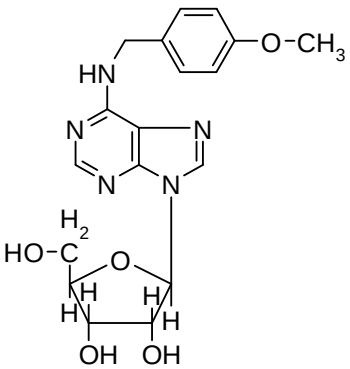
Me*p*T



Me*m*TR



Me*o*TR



Me*p*TR

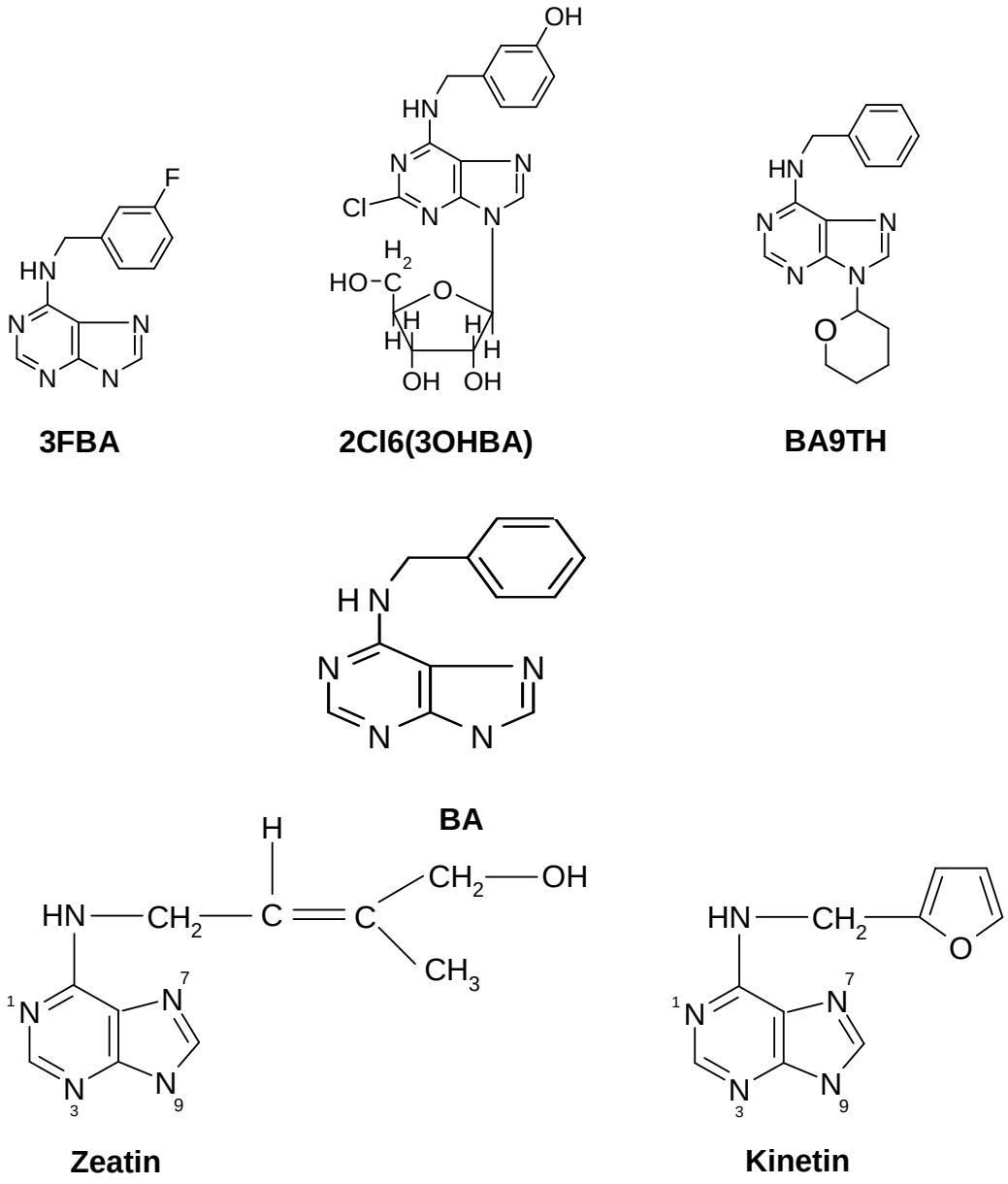


Figure 3.2: Molecular structure of the cytokinins tested in the soybean callus bioassay. Please note that compounds *o*TR and *p*TR were not studied but included for comparison.

4. Micropropagation and hyperhydricity in *Aloe polyphylla*

4.1 Introduction

Aloe polyphylla Schonland ex Pillans, commonly called spiral aloe, is a member of the Asphodelaceae. It is a beautiful threatened plant species native to the Maluti Mountains in Lesotho (Southern Africa). The Thaba Putsoa Range and the Maseru areas of the Drakensberg Mountains are the places where this species is found in greatest number (CHUKWUJEKWU *et al*., 2002). It grows in basalt rock crevices on high altitude slopes (2230 - 2720m) and is the only alpine member of the genus *Aloe*. It thrives well in loose rocks, which facilitate good drainage (AUBREY, 2002; CHUKWUJEKWU *et al*., 2002). This plant is grown mainly for horticultural (ornamental) and medicinal uses. Julian Mathews, garden and plant writer, describes this plant as "*an excellent example of a plant which is a living work of art - the spirals of closely packed, thick, fleshy leaves are a wonderful example of symmetry and the soft green colour is a delight"* MATHEWS, 2004).

The perfect spiral pattern in which the leaves are arranged is the most fascinating feature of this plant. Five ranks of leaves (containing 15-30 leaves each) form the spiral clockwise or anti-clockwise. Leaves are broad and have a grey-green colour with sharp tips, purplish brown in colour. It has approximately 75 – 150 leaves with irregularly toothed margins (EMANOIL, 1994; AUBREY, 2002). This species has attractive flowers with varied colour from dull red to salmon – pink and rarely yellow. Inflorescence is branched with a compact flower head. Flowering occurs from August (spring) through to December, peaking in September and October (summer) (EMANOIL, 1994).

Both sexual (seed) and asexual (vegetative) propagation are possible. Despite the production of large numbers of seeds, there is limited viability due to the endangered pollinator. Vegetative propagation by offshooting of pups is also very rare. To ensure

conservation of this species a micropropagation protocol has been developed (ABRIE and VAN STADEN, 2001; CHUKWUJEKWU *et al*., 2002). Incidence of hyperhydricity, the need for an additional rooting step and the use of zeatin (a very expensive cytokinin) are limitations of these protocols.

Currently benzyladenine is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability. It, however, has disadvantages in some crops. In micropropagated *Spathiphylum floribundum* [9G]BA accumulated at the base of the plant (WERBROUCK *et al.*, 1995). A slow release of this BA derivative caused heterogeneity in growth and inhibition of rooting during acclimatization. BA is also reported to causes hyperhydricity in many species (LESHEM and SACHS, 1985; LESHEM *et al*., 1988; TERAMOTO *et al*., 1993). Therefore, it is imperative to try and find an alternative to BA while maintaining reasonable multiplication rates and acceptable plant quality.

When WERBROUCK *et al.,* (1996) compared the types and effects of BA and *m*T derivatives in tissue culture of *S. floribundum,* they found that the main metabolite of BA, [9G]BA, was more stable but had a negative impact on rooting and acclimatization while, with the main metabolite of *m*T, mTOG, was easily degraded during acclimatization. When comparing the *post vitro* effect of different concentrations of BA and *m*T on rooting after an acclimatization period of four weeks, plants treated with *m*T produced a significantly higher number and greater length of roots than those treated with BA. Addition of the aromatic cytokinin *m*TR to the culture medium of potato significantly improved its survival in culture (BAROJA-FERNANDEZ *et al.,* 2002). KAMINÉK *et al*. (1987b) compared the activities of BA and *m*T in the induction of growth of lateral buds in *Poinsettia* and gerbera daisy and found that *m*T was more active. These results indicate that the slight structural difference between BA and *m*T could have a profound impact on plants during micropropagation. The aim of this section of the research project was therefore, to investigate the role of topolins in the micropropagation of *A. polyphylla* and control of hyperhydricity.

4.2 Material and methods

4.2.1 Source material and bulking of explants

In vitro grown cultures of *A. polyphylla*, initiated from seeds, were obtained from the Research Centre for Plant Growth and Development, University of KwaZulu-Natal Pietermaritzburg, South Africa. Bulking of stock material was done using full strength MS media (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol, 0.5 mg l^{-1} IBA, 1.0 mg l^{-1} zeatin and solidified with 1% agar (Bacteriological agar Oxoid Ltd., Basingstoke, Hampshire, England). Cultures were incubated in a growth room with continuous cool fluorescent tubes (Osram L75 W/20X) with a light intensity of 45 µmol m^{-2} s^{-1} and a temperature of 24±1ºC for nine weeks (CHUKWUJEKWU *et al*., 2002).

4.2.2 Shoot multiplication

After bulking sufficient explants, experiments to investigate the effect of *meta*-topolins on shoot multiplication were designed. The *meta*-topolin derivatives *m*T, Me*m*T and Me*m*TR along with BA and zeatin were investigated. The same type of growth media as used for bulking was used for the multiplication experiments. Concentrations 0.5, 2.5, 5.0, 7.5 and 15.0 µM were tested with five replicates and the control plants cultured with no cytokinin. Five shoot tip explants with 2-4 young leaves attached were cultured in screw cap jars (300 ml) containing 50 ml of media. A total of 25 explants per treatment were used. Cultures were then incubated under the same growth conditions mentioned above. After nine weeks, growth parameters including total number of shoots, multiplication rate, multiplication rate for shoots >1.5 cm, number of hyperhydric shoots, number of normal shoots, number of shoots >1.5 cm, number of shoots <1.5 cm, fresh weight, rooting ability (scored as 0 = absence, 1 = moderate rooting and 2 = excessive rooting) and leaf growth (scored as 1 = reduced leaf growth, 2 = average leaf growth and 3 = excessive leaf growth) were measured. The experiment was repeated three times.

4.2.3 DNA extraction and quantification

To study the effect of hyperhydricity on DNA synthesis, hyperhydric and normal shoots were produced using the optimized protocol. Induction of hyperhydricity was achieved by replacing the agar with gelrite and keeping the other media components the same. For observation purposes, two sets of cultures were prepared using BA and *m*T as cytokinins and agar and gelrite as gelling agents. DNA extraction and quantification was done by the protocols outlined in Appendix 2. Ten samples each of normal and hyperhydric plants were used. Results on ratio and DNA yield were then subjected to analysis of variance (GenStat 9th edition).

Before the start of DNA extraction and quantification the following assumptions were made to help explain and validate the results;

1. Since equal mass of tissue was used for DNA extraction variation in DNA quantity could be due to the inherent high water content of hyperhydric shoots; and
2. If assumption one is true, the difference between fresh and dry weight of both normal and hyperhydric samples should fully or partly explain the difference in DNA yield. To support this hypothesis equal mass of tissue of normal and hyperhydric plants treated with *m*T (with agar and gelrite respectively) was taken at the time of DNA extraction. Fresh weight and dry weight was then compared and the difference in change in mass between the normal and hyperhydric samples was noted;

The remaining approximately 50 plants of each of normal and hyperhydric cultures treated with BA and *m*T were left in the culture room for observation as to their capacity to recover and to see if the plants were able to reverse hyperhydricity without additional treatment.

4.2.4 Acclimatization

On the basis of results of the multiplication experiments, only those treatments that gave acceptable multiplication rates and the optimum cytokinin concentration that induced better morphogenesis were considered for the acclimatization experiments. Plants treated with 2.5 and 5.0 µM of *m*T and zeatin and 2.5 µM BA were used. BA failed to produce healthy shoots but was included for comparison purposes as it is the most widely used cytokinin in tissue culture. Shoot clusters were carefully separated from each other in such a way that all had at least one functional root. Individual plants were then washed thoroughly and extra large roots cut back. Plants were planted in a potting mixture with a 1:1:1 ratio of soil, sand and vermiculite, treated with fungicide (Benlate, 0.01%) and transferred to a mist house with 90% relative humidity. After four weeks in the mist house, plants were transferred to a greenhouse in the same potting mix (CHUKWUJEKWU *et al*., 2002). After two months of growth in the greenhouse, fresh weight of shoots and roots was analyzed (GenStat 9th edition).

4.3 Results and discussion

4.3.1 Effect of *meta*-topolin on shoot multiplication

The choice of cytokinin to use in tissue culture is determined by its cumulative efficiency in inducing an acceptable rate of shoot multiplication, normal shoots and roots and the eventual ability of plants to acclimatize easily. The shoot and root growth responses and variations observed and analyzed in this study are due to treatment effects as indicated in Table 4.1. Means of total number of shoots showed significant differences among the different cytokinins and concentrations tested. At lower concentrations, BA produced more shoots. As the concentration increased larger numbers of shoots were recorded with the *meta*-topolin treatments (Figure 4.1 A-C). Both total multiplication rate (Figure 4.1 B) and multiplication rate of shoots greater than 1.5 cm in length (Figure 4.1C) were recorded and analyzed separately.

Results of the analysis showed that treatment means were significantly higher than the control for most cytokinin levels tested. Multiplication rates increased with an increase in concentration up to 15.0 µM, where a decline in multiplication rate and excessive abnormal growth were observed. A better multiplication rate, spontaneous rooting and healthy explants were found at 5.0 µM level for the *m*T treatment (the quality not found in any of the other treatments), hence this was selected as the optimum cytokinin level. At this level a multiplication rate of eight shoots (shoots greater than 1.5 cm in length) per explant was found with the *m*T treatment. This rate was significantly higher than all the other treatments and better than previous reports of a total of seven shoots per explant (CHUKWUJEKWU *et al*., 2002). A few reports on the use of topolins indicate that this group of cytokinins could be a new source of cytokinins with high morphogenetic activity. KAMINEK *et al*. (1987b) found that *m*T was nearly twice as effective as BA in the induction of shoot growth of cuttings. KUBALAKOVA and STRNAD (1992) compared the effects of aromatic and isoprenoid (zeatin) cytokinins on micropropagation and organogenesis of sugar beet cultures and found higher activity (greater number of shoots per explant) with *m*T. They also observed abnormal growth in BA-treated plants during subculturing, but not with *m*T.

Table 4.1: The effect of various cytokinin treatments on overall shoot and root growth. Observations for the control had no variation among replicates and hence the statistical test was not applied.

Treatments (µM concentration)	Shoot growth		Root growth	
	Pearson chi-square value	Probability level	Pearson chi-square value	Probability level
control	-	-	-	-
0.5	31.50	<0.001	17.53	0.063^{ns}
2.5	35.56	<0.001	24.00	0.008
5.0	30.77	<0.001	34.29	<0.001
7.5	19.44	0.035	40.80	<0.001
15.0	28.26	0.002	43.20	<0.001

^{ns} indicates non-significant treatment effect. This table shows that the observed differences in the parameters analysed are due to treatment effect.

The use of *m*TR for improving survival of potato cultures has been reported (BAROJA-FERNANDEZ *et al*., 2002). It was also observed that *m*TR (which was not included in this experiment) had a comparable effect with that of *m*T when applied at higher concentrations than the optimum level of *mT* (5 µM) in *A. polyphylla*. Between 5-15 µM concentrations *m*TR produced a comparable number of shoots and improved the rooting ability of the plants (with longer duration in culture) unlike *m*T, which caused inhibition of rooting and abnormal growth at concentrations greater than 5 µM (Figure 4.2 I). *m*TR was tested after the whole experiment was completed.

The slight structural difference between BA and *m*T (Figure 3.2) had a notable effect on cultures of *S. floribundum* (WERBROUCK *et al*., 1996). In this study, it was found that *A polyphylla* plants treated with *m*T were superior in quality and quantity compared to plants treated with BA (Figure 4.2) indicating that *meta*-topolin would be a good replacement for zeatin in the tissue culture of *A. polyphylla*. Given the structural difference between zeatin and *m*T, an explanation of this result would be the pattern of receptor recognition as position specificity is related to receptor recognition (MOK *et al*., 2005). In cytokinin recognition studies using the *Arabidopsis* CRE1, WOL, AHK4 and maize ZmHK1 receptors, the authors found that AHK4 responded to *trans*-zeatin and *m*T while ZmHK1 responded to *cis*-zeatin and *o*T (MOK *et al*., 2005). This similar affinity in receptor recognition could be the possible reason why *m*T and zeatin had comparable effects in tissue culture of *A. polyphylla*. Spontaneous rooting of plants in multiplication media, a better multiplication rate and lower cost however, make *m*T the preferred cytokinin.

4.3.2 Effect on hyperhydricity

Generally, the incidence of hyperhydricity increased with an increase in the concentration of cytokinins (Table 4.2), a fact well documented in the literature. At an optimum concentration (5.0 µM), no hyperhydric shoots were recorded for the *m*T and Me*m*T treatments. Hyperhydricity was most severe with BA treatments although all treatments caused hyperhydricity at higher concentrations (>5 µM). Although there

was not a significant difference among the treatments (except for BA), the higher multiplication rate and good rooting in a multiplication media makes *m*T the preferred cytokinin. Apart from hyperhydricity, BA-treated plants failed to root, were yellowish in colour and had excessive abnormal shoot growth (Figure 4.2). Type of gelling agent and cytokinin play a major role in the control of hyperhydricity in *A. polyphylla* with BA and gelrite causing the most hyperhydricity.

4.3.3 Effect on fresh weight (FW)

Total fresh mass per jar was measured and analyzed. There was a strong treatment effect at all concentrations for all the cytokinins tested. Mean mass of treatments were significantly higher than the control. At lower concentrations, BA and zeatin gave higher FW. Unlike other treatments where a decline was observed at higher concentrations, (Table 4.3) there was a consistent increase of FW with *m*T treatment. At optimum concentration (5 µM) zeatin gave significantly higher FW but had lower multiplication rates compared to *m*T (Figure 4.4). This indicated that FW was not a reliable measure of multiplication and normal growth as it was affected more by growth of individual plants than by multiplication rate. It has also been observed that hyperhydricity and an undifferentiated mass of tissue due to abnormal growth contributed to an increase in FW.

At higher cytokinin concentrations, plants treated with BA and zeatin had lower multiplication rates, more pronounced abnormal growth and smaller mass of fresh weight compared to plants treated with *m*T (Tables 4.2 and 4.3). This indicates that at higher equimolar concentrations *m*T was less toxic to *A. polyphylla* compared to BA and zeatin (see Figure 4.2 G and H for comparison between BA *m*T). KAMINEK *et al*. (1987b) reported that *m*T promoted shoot formation of stem cuttings of *Ephorbia pulcherrima* Wild. and *Gerbera jamesonii* Hook at 10^{-4} mol L^{-1} concentration. At this concentration BA inhibited shoot formation. They attributed this effect to the faster translocation of *m*T in plant tissues which prevents its localized accumulation.

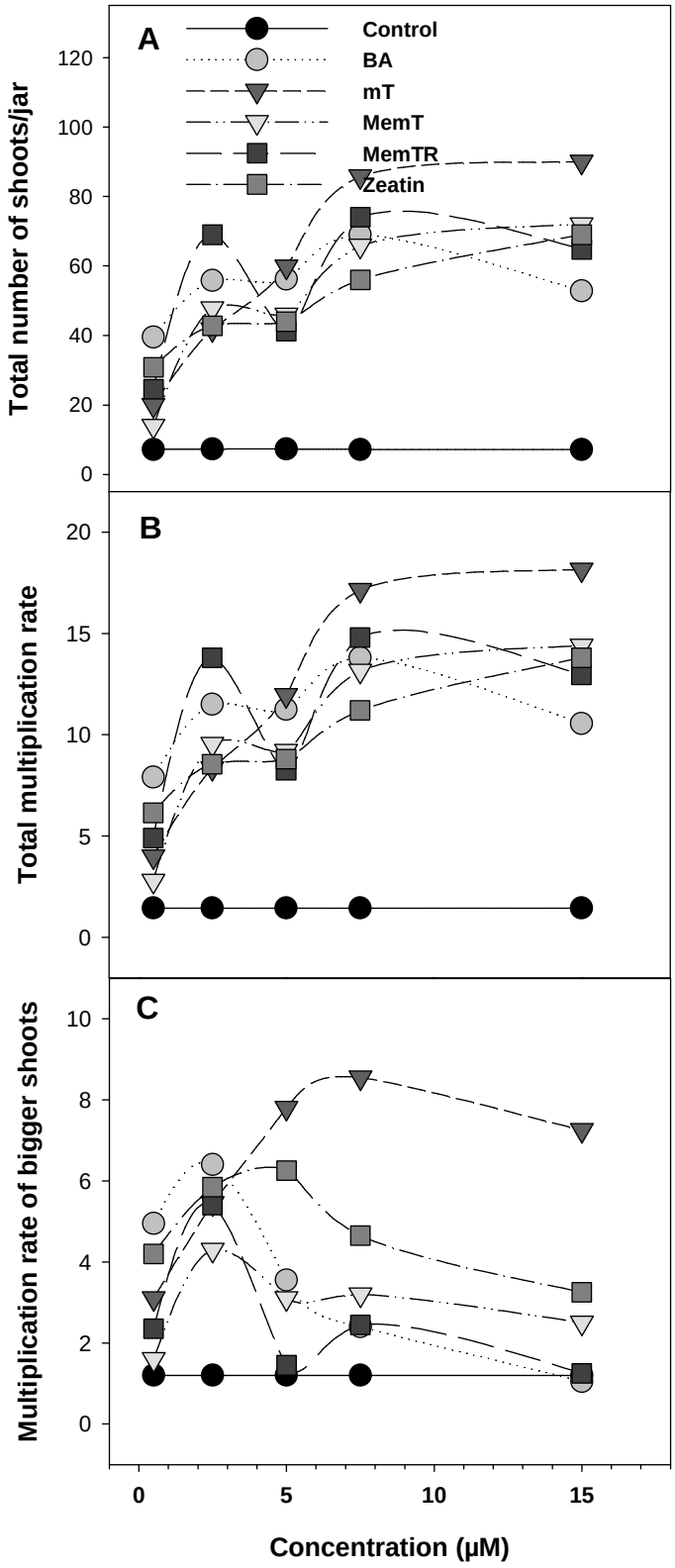


Figure 4.1: The effect of the type and concentration of cytokinin on shoot multiplication (A), total multiplication rate (B) and multiplication rate of shoots greater than 1.5 cm in length (C). Note that *meta*-topolin gave more shoots per jar and a larger number of shoots big enough for acclimatization. The 5 µM *m*T treatment was selected

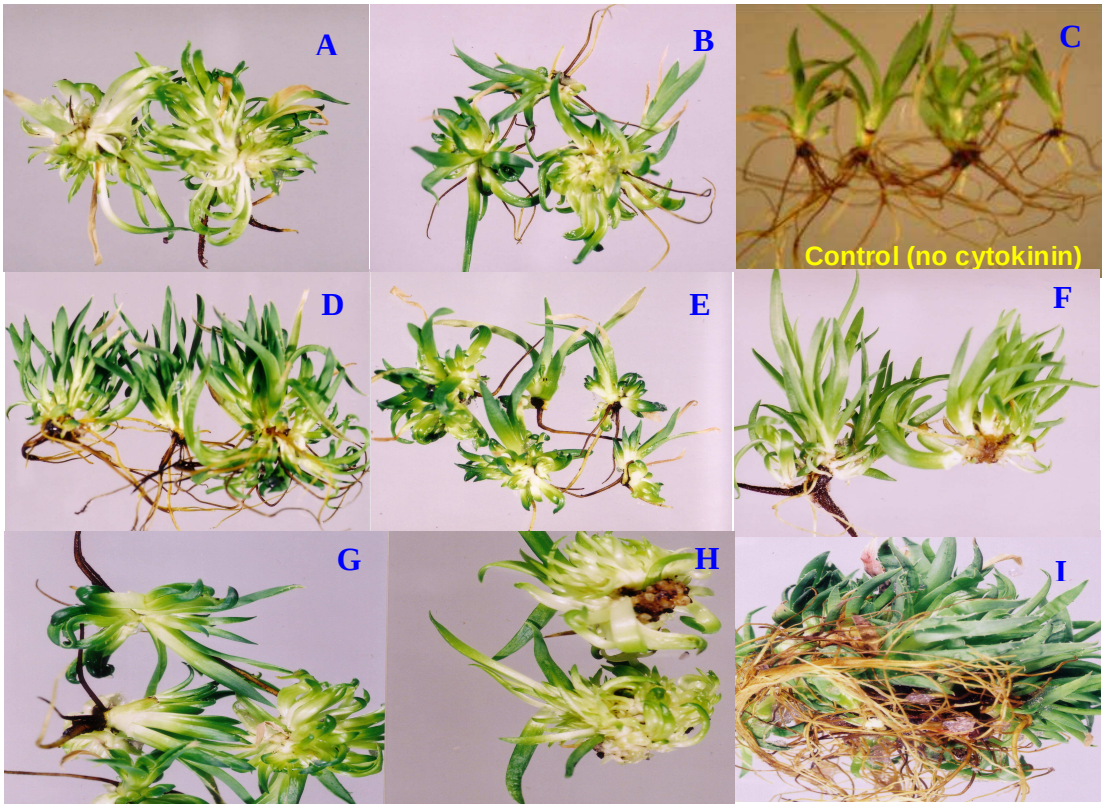


Figure 4.2: Effect of the various cytokinin treatments on shoot and root growth and incidence of abnormality of *A. polyphylla* (A = BA; B = Me*m*T; C = Control; D = *m*T; E = Me*m*TR; F = Zeatin; G = 15 µM *m*T, H = 15 µM BA and I = 7.5 µM *m*TR). Note the *m*T-treated plants had healthy shoot growth and numerous roots as opposed to the abnormal growth (BA) and failed rooting (zeatin). Also note the relatively less toxic effect of *m*T compared to BA (G and H)

Table 4.3: Effect of type and concentration of cytokinin on mean fresh weight (g) of *A polyphylla*

Treatments	Cytokinin concentration (µM)				
	0.5	2.5	5.0	7.5	15
Control	4.00±0.46	4.00±0.46	4.00±0.46	4.00±.46	4.0 0±.46
BA	17.52±0.84	18.85±1.95	15.27±1.4	11.86±1.62	14.98 ±1.33
*m*T	11.16±2.20	15.6±1.66	17.75±2.04	20.58±0.62	22.96± 1.08
Me*m*T	9.11±2.16	10.77±0.46	8.88±1.17	9.84±1.85	14.55±1. 60
Me*m*TR	12.10±1.86	11.84±1.76	7.54±1.48	9.12±1.86	11.37± 1.81
Zeatin	16.85±2.32	18.76±1.2	22.06±1.05	16.71±1.25	1 6.55±.026
LSD (5%)	**3.564**	**4.410**	**3.729**	**4.281**	**3.334**
F Probability	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

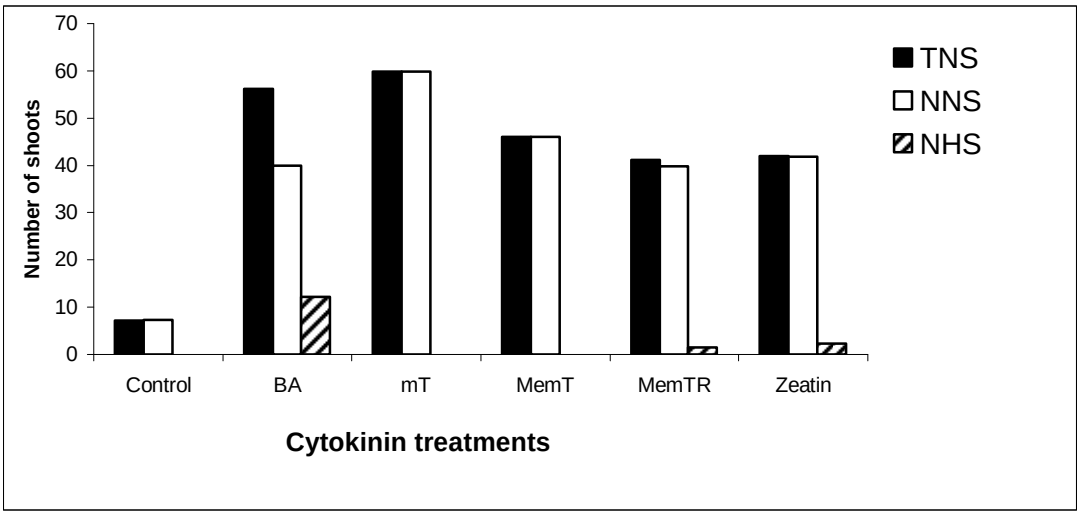


Figure 4.3: The effect of the selected optimum cytokinin concentration (5 µM) on hyperhydricity of *A polyphylla* cultures. TNS = total number of shoots; NNS = number of normal shoots; NHS = number of hyperhydric shoots. Note that hyperhydricity was totally controlled using *m*T. See Table 4.2 for statistical details.

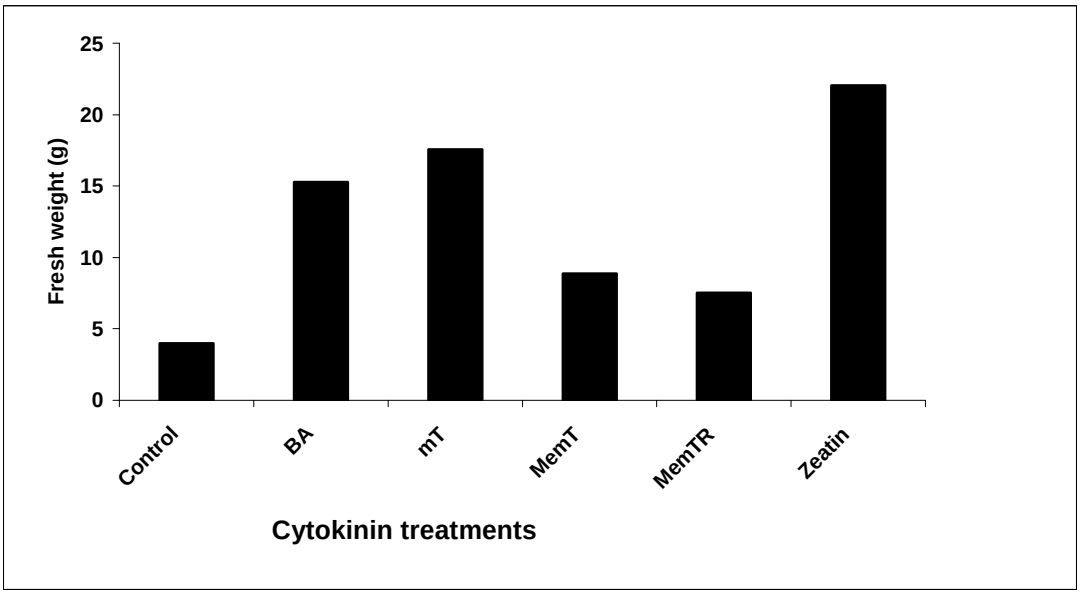


Figure 4.4: Effect of the selected optimum cytokinin concentration on fresh weight of *A polyphylla* cultures. See Table 4.3 for statistical details.

4.3.4 Effect on acclimatization *ex vitro* on *A. polyphylla*

More than 91% of the plants treated with *m*T acclimatized successfully compared to 65% survival rate recorded with BA-treated plants (Table 4.4). Results *ex vitro* showed that the effect of *m*T is not only limited to *in vitro* growth with significant treatment effects noted during the acclimatization process. Acclimatized plants treated with *m*T showed faster growth and superior rooting compared to BA and zeatin-treated plants as indicated by mean fresh weight of roots and shoots (Table 4.5). Under normal conditions of growth, root and shoot growth complement one another. The poor *ex vitro* growth observed with plants treated with BA and zeatin (although statistically not significant when zeatin was compared to *m*T) could therefore be attributed to a treatment effect. The negative effect of BA on *ex vitro* growth could be due to the accumulation of the more stable derivative, [9G]BA. Accumulation of [9G]BA on the basal portion of *Spathyphylum* plants resulted in slow release of BA during acclimatization, which caused different problems such as heterogeneity in growth and inhibition of rooting. The concentration of [9G]BA was about ten times more than the BA concentration in the medium after just two weeks of growth. Apart from its high concentration, this derivative is produced and stored at the plant base rather than being transported and could have contributed to its negative effect (WERBROUCK *et al*., 1995).

4.3.5 Effect of hyperhydricity on total DNA content of *A polyphylla*

The effect of hyperhydricity on the quality and quantity of DNA was studied to verify if this abnormality affects DNA synthesis. Results of the experiment indicated that hyperhydric samples produced three times less DNA than the normal samples (Table 4.6). This result can be explained based on two assumptions. Firstly, low DNA levels could be due to the high level of water content in hyperhydric shoots which could have affected the DNA yield. Secondly, it could be as a result of a negative effect of hyperhydricity on DNA synthesis.

Table 4.4: Effect of various cytokinin treatments on acclimatization of *A polyphylla*. *Aloe polyphylla* can easily be acclimatized to *ex vitro* growth conditions.

Treatments	Total number transferred to mist house	Total number survived in the greenhouse	Survival rate (%)
2.5 BA	45	30	66.67
2.5 *m*T	45	42	93.33
2.5 zeatin	45	39	86.67
5.0 *m*T	45	41	91.11
5.0 zeatin	45	38	84.44

Table 4.5: Effect of the different cytokinins tested on *ex vitro* growth. Fresh weight of ten (random) fully acclimatized (two-month-old) plants per treatment was used for this analysis. Only those treatments that produced plantlets with both shoots and roots were considered.

Cytokinin concentration (µM)	Mean fresh weight (g)	
	Shoot	Root
2.5 BA	5.9±1.37	0.56±0.12
2.5 *m*T	19.3±2.83	1.75±0.26
2.5 zeatin	13.2±2.23	1.00±0.14
5.0 *m*T	23.6±7.03	1.66±0.44
5.0 zeatin	14.9±2.65	0.82±0.17
LSD (5%)	**10.87**	**0.726**
F Prob.	**0.028**	**0.006**

The first assumption failed to fully explain the result since the difference in change in mass between fresh and dry weight of the normal and hyperhydric sample was less than two fold leading to the conclusion that hyperhydricity does affect DNA synthesis. Very little has been done on the molecular biology of hyperhydricity, especially on aspects of DNA synthesis. Currently, there is only one report which demonstrated that hyperhydricity is linked with abnormal DNA content in grass pea *Lathyrus sativus* L. (OCHATT *et al*., 2002).

Table 4.6: Effect of hyperhydricity on DNA quantity and quality

Treatments	Ratio ($A_{corrected\ 260}/A_{280}$)*	DNA yield (µg per g tissue)
Normal plants	1.66±0.033	119.0±9.43
Hyperhydric Plants	1.44±0.032	35.8±3.46
S.E.	**0.0467**	**10.05**
F probability	**< 0.001**	**< 0.001**
LSD	**0.098**	**21.11**

* see appendix 2 for details of DNA quantification procedures.

The significantly poor quality of DNA in the hyperhydric plants, as indicated by the ratio (Table 4.6), could be due to the excess water in the leaf tissues of hyperhydric plants and/or the production of some metabolites by the plant in response to hyperhydricity, that could interfere with the DNA extraction. It should therefore be noted that a more stringent approach should be followed while extracting DNA from hyperhydric samples as the protocol developed for normal samples may not always produce high quality DNA from hyperhydric plants.

Results of the observation trial on inherent reversal of hyperhydricity revealed that the ability of plants to reverse hyperhydricity was affected by the type of cytokinin. Keeping other media components the same, *m*T-treated plants showed significant recovery and started producing new healthy shoots (with extended duration in culture) as opposed to BA-treated plants which completely failed to reverse hyperhydricity, turned yellow and eventually died. This could be due to the more stable nature of BA and its metabolites which tend to stay longer in the plant tissue than *m*T (WERBROUCK *et al*., 1995). The soybean callus bioassay (Figure 3.1, chapter 3) also revealed that BA is more toxic than *m*T. This toxicity coupled with its inherent stability could have contributed to the failure to recover of the BA-treated plants.

5. Shoot-tip necrosis in *Harpagophytum procumbens*

5.1 Introduction

Indigenous to southern Africa, *H. procumbens* is a member of the Pedaliaceae family. It is weedy perennial tuberous plant with creeping annual stems of up to two metres long. The above ground parts emerge after the first spring rain and die back during winter or drought. Commercial devil's claw (commonly used name for commercial *Harpagophytum species*) comprises two species namely *H. procumbens* and *H. zeyheri*, each having different sub-species (Table 5.1). In addition to the name 'devil's claw' which represents the two species of the genus *Harpagophytum*, other common names such as grapple plant, wood spider, duiwelsklou, teufelskralle, griffe du diable, sengaparile, kamangu, kanako and other local names are also used. The subject of this study, *H. procumbens,* is commonly traded under the pharmaceutical name Harpagophyti radix (STEWART and COLE, 2005).

Table 5.1: Sub-species of the two commercial *Harpagophytum* species. Differences between taxa are based on flower and fruit characteristics (STEWART and COLE, 2005)

H. procumbens sub-species	*H. zeyheri* sub-species
procumbens (Burch) de Candolle ex Meissner	*schiffii* Ihlenfeldt and H. Hartmenn
transvaalense Ihlenfeldt and H. Hartmenn	*sublobatum* (Engler) Ihlenfeldt and H. Hartmenn
	zeyheri Decaisne

Harpagophytum spp. belong to some of the most studied medicinal plants with pharmacological activity analysis and with clinical tests dating back to the 1960s. Extracts of tubers of *Harpagophytum spp*. are active in the treatments of degenerative rheumatoid arthritis, osteoarthritis, tendonitis, kidney inflammation and heart disease (STEWART and COLE, 2005 and references therein). A large supply of these plants for international trade come from Namibia with South Africa and Botswana also

contributing their share (RAIMONDO and DONALDSON, 2002). Despite the huge economic importance, the cultivation of these species is facing some problems. Attempts to commercially propagate these species from seeds failed due to low germination rates. Plants propagated by cuttings failed to produce primary roots resulting in a single harvest (KATHE *et al*., 2003).

An *in vitro* propagation method of Devil's claw was reported (LEVIEILLE *et al*., 2000; LEVIEILLE and WILSON, 2002) and gave a coefficient of multiplication of four plants per month per explant. This technique, using single node explants, consisted of root induction treatments with IAA, in the dark, followed by plant development in growth regulator-free medium, under light (LEVIEILLE and WILSON, 2002). An *in vitro* method was also developed in Pietermaritzburg, South Africa by the Research Centre for Plant Growth and Development where a plant development step using BA as the sole growth regulator was included. Developed plantlets spontaneously rooted in the same medium, after two months of culture. This second method seems attractive as it eliminates sub-culturing to rooting medium. However, shoot-tip necrosis (STN) occurred during elongation of the explants. This rapid necrosis of the apical shoot triggered growth of axillary buds. This mode of development produced plants with many branches before acclimatization and transplantation into soil. With longer duration in culture and repeated sub-culturing, the problem of STN became severe with plants accumulating basal callus and eventually losing their ability to root in a multiplication medium (RAMAROSANDRATANA, personal communication).

Apical shoot necrosis has been reported due to pathogenic infection (BENTUR and KALODE, 1996; GUTHRIE *et al*., 2001) and calcium deficiency (MORARD *et al.,* 1996; SELING *et al*., 2000). However, *H. procumbens* naturally shows dieback of shoots between April and June as a prelude to winter dormancy (RAIMONDO and DONALDSON, 2002). The problem of non-pathogenic dieback or shoot necrosis *in vitro* has been associated with various culture conditions such as salt formulation and growth regulators (VIEITEZ *et al.,* 1989; KATAEVA *et al.,* 1991; MACKAY *et al.,* 1995; MORARD *et al.,* 1996; PIAGNANI *et al*., 1996; GRIGORIADOU *et al.,* 2000;

PEREZ-TORNERO and BURGOS, 2000; SELING *et al*., 2000), use of activated charcoal (MACKAY *et al.*, 1995; WANG and VAN STADEN 2001), duration in culture (GRIGORIADOU *et al.,* 2000), type of tissue culture medium (GENEVE *et al*., 1992; MACKAY *et al.,* 1995; GRIGORIADOU *et al.,* 2000), exudation of phenolics in tree species (BELLAROSA, 1988), physiological disorder associated with rooting (KATAEVA *et al.,* 1991), sulphur content and NH_4/NO_3 ratio (LAKSHMI and RAGHAVA, 1993), fructose (KULKARNI and D'SOUZA, 2000) and pH flactuations (De BLOCK, 1990). These factors are discussed in detail in Chapter Two Section 2.3.

5.2 Materials and methods

Previously maintained cultures of *Harpagophytum procumbens* were used as the source of nodal explants. Cultures were sub-cultured to fresh medium after every 3-4 weeks to maintain the stock. Cultures were incubated in a growth room with cool fluorescent tubes (Osram L75 W/20X) with a light intensity of 45 µmol m^{-2} s^{-1} and a temperature of 24±1ºC at 16-hour light and 8-hour dark photoperiod. The nodal explants (1 cm) were implanted into medium supplemented with 0.9 % (w/v) agar and 3% (w/v) sucrose. These culture conditions were also used for all experiments.

5.2.1 Effect of media type and strength on STN

To investigate the effect of type and strength of nutrient media on STN, MURASHIGE and SKOOG (1962), NITSCH and NITSCH (1969) Basal Medium (NB) and Woody Plant Medium (WPM) (LLOYD and McCOWN, 1980) supplemented with 0.9 % (w/v) agar, 3% (w/v) sucrose and 1.0 mg l^{-1} BA and were tested in a completely randomized design at three levels; full strength, half-strength and quarter-strength. The pH of the media was adjusted to 5.8 with 1N HCl or 1 N NaOH, before addition of agar. Thirty ml of medium was poured into 250 ml screw cap jars which were then autoclaved at 1.05 KPa at 121 °C for 20 min. Cultures were maintained in the conditions described above. Each treatment had four replicates with six nodal explants each. After four weeks of growth, growth parameters such as average

number of shoots per explant, percent necrotic shoots and average shoot length were measured and analyzed using the MINITAB version 14 (Minitab Ltd., USA). Fisher's least significant difference (LSD) at the 5% level was used to analyze the differences between the means.

5.2.2 Effect of type and concentration of cytokinins on STN

The effect of cytokinins on STN was assessed using half strength MS media containing media supplements indicated in section 5.2.1 above. Initially BA and kinetin (Sigma-Aldrich) were tested at various concentrations (2.22, 4.44, 6.66 and 8.88 µM) and the control plants were cultured in hormone free half-strength MS media. Each treatment had four replicates with six nodal explants each. After four weeks of growth, growth parameters such as average number of shoots per explant, percent necrotic shoots and average shoot length were measured and analyzed using the MINITAB version 14 (Minitab Ltd., USA). Fisher's least significant difference (LSD) at the 5% level was used to analyze the differences between the means.

In an attempt to find an alternative to BA and kinetin, the effect of *m*T and *m*TR with and without auxin on STN was also investigated and results analysed using SPSS package version 15.0. Cultures from these experiments were also used to assess the effect of sub-culturing. Uniformly grown cultures were divided into two categories. Cultures in the first category were sub-cultured every four weeks whereas cultures in category two were sub-cultured every two weeks, keeping other media components constant. Growth parameters were then recorded and analysed.

5.2.3 Effect of type and concentration of sugars on STN

Various concentrations of sucrose (1, 2, 3 or 4%) and equi-molar concentrations (0.086 M) of different sugars (glucose, fructose, sucrose or maltose) were also tested. A completely randomized block design with four replicates having 25 explants each was used. The culture responses were expressed in terms of number of shoots per

explant, percentage of necrotic shoots and average length of shoots. Percentages of response data were analyzed using one-way analysis of variance (ANOVA) after arcsine transformation using the MINITAB package.

5.2.4 Effect of calcium and boron on STN

To assess the effect of calcium and boron on STN, an experiment having three steps was designed. Treatments in step one contained constant boron and varied levels of calcium (6, 9, 12, and 15 mM), step two contained constant calcium and varied levels of boron (0.2, 0.3, 0.4 and 0.5 mM) and step three had proportionally varied levels of both calcium and boron (6 mM Ca + 0.2 mM B, 9 mM Ca + 0.3 mM B, 12 mM Ca + 0.4 mM B and 15 mM Ca + 0.5 mM B). The concentrations of both elements in MS media (3 mM for Ca and 0.1 mM for B) were taken as standard concentrations. The control plants were cultured in full strength MS media. Other media components were kept constant. Each treatment had four replicates with 40 ml of media having 6 explants per screw cap jar each. After four weeks of growth, growth parameters such as average shoot length per jar (cm), fresh and dry weight (g) and average number of infections per jar were recorded and analysed using SPSS version 15.0 and mean separation by least significant difference (LSD) was done using Duncan's Multiple Range Test.

5.2.5 Rooting and acclimatization of tissue cultured *H. procumbens*

Based on the results of the above experiments, a protocol to successfully multiply the plants was established. This protocol involved the use of auxin-free half strength MS media with 6 mM calcium, 3 % sucrose, 0.1 g l^{-1} myo-inositol and 5 µM *m*TR solidified with 1 % agar after adjusting the pH to 5.8. Forty ml of the media were added to 250 ml screw-cap jars after autoclaving. Six to ten nodal explants per jar were used without notable effect on growth and multiplication. Regenerated plantlets were then transferred to cytokinin-free rooting media containing half strength MS media supplemented with 2.5 µM IAA and the same supplements as above. The transfer to

rooting media, was preceded by careful trimming of the basal callus, after three weeks when the first symptom of tissue browning was observed. This initial attempt to root plantlets was successful and hence no additional rooting experiment was done.

Two sets of rooted plantlets were then potted in 1:1 mixture of sand and soil for *ex vitro* acclimatization. The first set was transferred to the mist house and the second set transferred directly to the greenhouse. Fully acclimatized plants were then transferred to bigger pots containing the same potting mixture to evaluate their ability to produce tubers and left to grow for one year with a watering frequency of once per week.

5.3 Results and discussion

5.3.1 Effect of media composition and strength on STN

Three different basal media (MS, NB and WPM) were used for raising cultures of *Harphagophytum*. The three basal media differed in their ionic strength. Of the three media tried, MS exhibited the maximum (98 %) necrotic shoots while WPM had the least number (88 %) of necrotic shoots. Half-strength and quarter-strength of all three basal media resulted in less necrotic shoots compared to their full-strength counterparts.

Lowering the concentration of salts in media reduced the shoot elongation capacity. On the half- and quarter-strength WPM as well as on quarter-strength NB medium, shoot elongation was drastically affected. In some cases, the shoot buds failed to open even after a prolonged culture period of 8 weeks (Table 5.2). The number of shoots developed per explant varied significantly on full- and half-strength medium ($P \leq 0.05$).

Table 5.2: Effect of media composition and strength of media on STN of *H. procumbens*

Media	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
MS			
Full-strength	1.68±0.09[a]	88[a]	5.08±0.09[a]
Half-strength	1.59±0.07[a]	29[b]	5.08±0.10[a]
Quarter-strength	1.60±0.07[a]	14[c]	3.26±0.08[b]
NB			
Full-strength	1.64±0.07[a]	90[a]	3.69±0.09[b]
Half-strength	1.62±0.06[a]	27[b]	1.53±0.05[c]
Quarter-strength	1.64±0.07[a]	26[b]	*
WPM			
Full-strength	1.49±0.05[b]	86[a]	3.31±0.16[b]
Half-strength	1.43±0.04[b]	27[b]	*
Quarter-strength	1.23±0.06[c]	28[b]	*

* shoot buds failed to attain measurable length

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

5.3.2 Effect of cytokinins on STN

Plants cultured in media containing BA were affected by STN at all concentrations. The control plants (with no plant growth regulators) showed no symptoms of necrosis but failed to grow and remained stunted (Figure 5.1c). On kinetin-supplemented medium, a higher percentage of necrotic cultures were obtained compared to that of BA-supplemented media. Among the various concentrations of BA tested, 4.44 µM favoured maximum number of shoot development per explant, while 6.66 µM favoured the elongation of shoots.

The symptom of shoot-tip necrosis started randomly in all cultures after nine days in culture. With increased duration in culture, the number of plants affected increased and all plants were affected by the end of the sixth week. The severity of the problem

was also associated with cytokinin concentration. At higher concentrations there was a more pronounced death that included necrosis of most leaves. It was also observed that the death of the apical shoot resulted in a branched growth. It was after the death of the apical shoot that lateral shoots emerged. It appeared that the apical shoots were dying to allow the growth of the lateral shoots. The lateral shoot that developed first assumed the role of the apical shoot and was the next to be affected, with necrosis continuing hierarchically until all shoots were affected. Plants cultured on media containing both auxin and cytokinin and cytokinin alone showed necrotic symptoms unlike the control plants cultured in hormone free media. The problem was more severe when auxin was added to the media. Results also showed that STN is affected by the auxin:cytokinin ratio. A bigger ratio (more auxin) had a more pronounced effect. Treatments containing *m*TR had lower percentages of necrotic shoot-tips when compared with BA and *m*T (Table 5.4).

The use of cytokinin with or without auxin totally inhibited rooting. Plantlets also accumulated a mass of callus-like tissue at their base (Figure 5.1 A and B). The amount of this callus-like tissue varied among the treatments. It increased with an increase in cytokinin concentration and addition of IAA to the medium (Figure 5.2). This basal callus totally inhibited rooting, and it was an absolute requirement to trim off all basal calli for rooting to resume in a rooting medium. VIEITEZ *et al*. (1989) observed the development of basal callus on vigorously growing chestnut and oak cultures. They suggested that this basal callus may operate as a sink that traps some media components which otherwise would have helped the plant to overcome certain deficiency symptoms. The association of callus induction with the accumulation of Ca^{2+} was reported on melon cultivars (KINTZIOS *et al*., 2004). This report could partly explain the development of basal callus to be due to Ca^{2+} accumulation at the base of the plantlet, a phenomenon which may result in Ca deficiency in the upper part of the plantlets. Cytokinins play a role in the regulation of apical dominance and the transmission of nutritional signals (SAKAKIBARA, 2004). Any irregularity in the action and availability of cytokinins is, therefore, most likely to cause disruption in the nutrient balance of the whole plant.

Table 5.3: Effect of BA and Kinetin on growth and STN of *H. procumbens*

Treatment (µM)	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
1/2 MS	1.48±0.08^{e}	28^{d}	4.53±0.05^{b}
BA			
2.22	3.00±0.09^{c}	25^{d}	4.25±0.0^{b}
4.44	5.22±0.15^{a}	28^{d}	5.34±0.08^{b}
6.66	4.62±0.24^{b}	29^{d}	7.89±0.15^{a}
8.88	4.90±0.13^{b}	28^{d}	4.39±0.07^{b}
Kinetin			
2.22	1.00±0.18^{e}	57^{a}	3.65±0.02^{c}
4.44	1.56±0.27^{e}	49^{b}	3.68±0.02^{c}
6.66	1.96±0.21^{e}	39^{c}	3.78±0.03^{c}
8.88	2.38±0.19^{d}	35^{c}	3.73±0.03^{c}

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

5.3.4 Effect of sub-culturing on STN

Transferring of explants to a freshly prepared medium reduced the development of necrotic shoots. A significant decline in the percentage of necrotic cultures was recorded (Table 5.5) following the transfer of explants to fresh medium after 2 weeks, compared to those maintained on the same medium for 4 weeks. This approach also favoured both proliferation and elongation of shoots. MNG'OMBA *et al*., (2007) reduced the effect of STN on jacket plum by continuously sub-culturing microshoots to fresh medium. They noted that the use of 0.3 mg l^{-1} casein hydrolysate slightly improved STN. However, this had a negative effect on cultures of *H. procumbens* (results not presented).

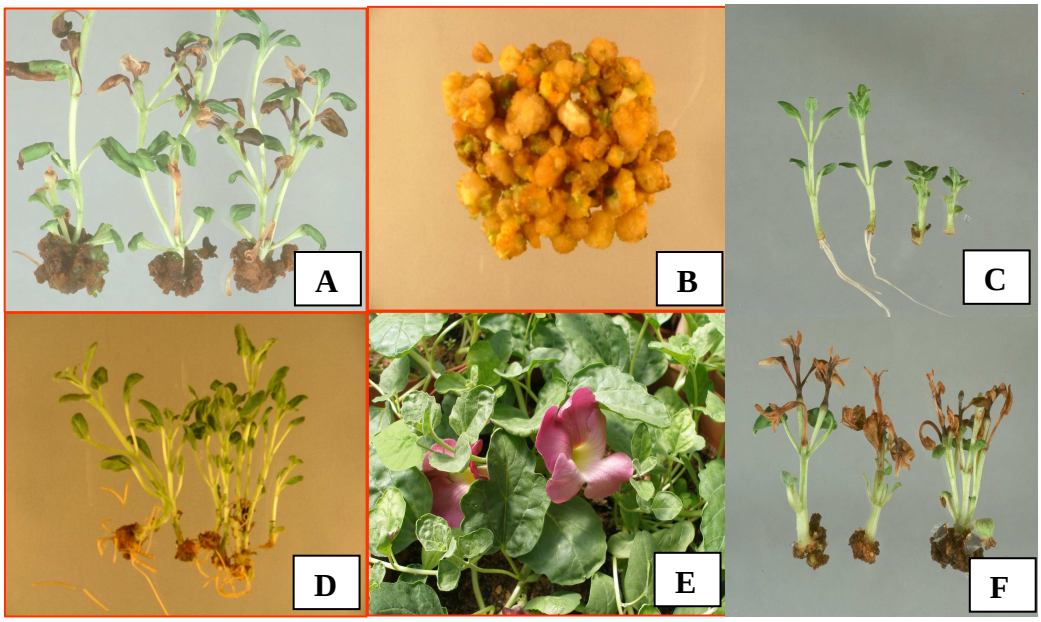


Figure 5.1: *In vitro* grown *H. procumbens* (A) necrotic symptoms and development of basal callus on four-week-old culture; (B) trimmed basal callus; (C) two-month-old plantlets cultured in hormone free medium; (D) rooted plants ready for acclimatization; (E) fully acclimatized and greenhouse-grown plants and (F) example of STN at its worst.

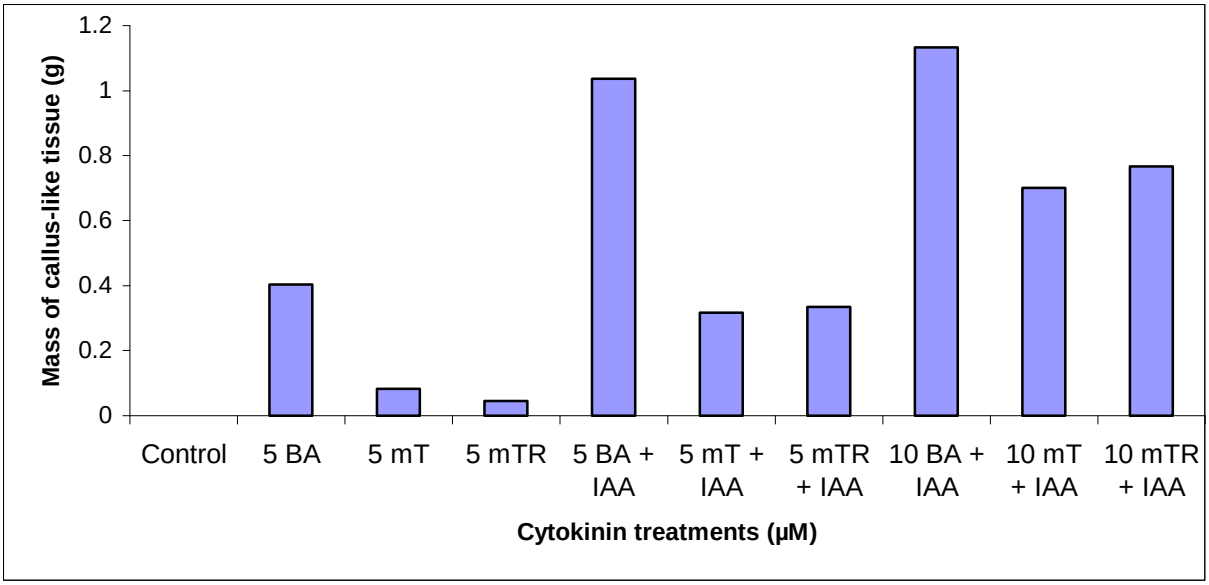


Figure 5.2: The effect of BA, *m*T and *m*TR (5 and 10 µM) with (2.5 µM) and without IAA on the development of basal callus

5.3.5 Effect of sucrose concentration and type of sugars on STN

Sucrose is an important source of carbohydrate in the culture medium. It also acts as an osmoticum. Various concentrations of sucrose were tried. At 1% level, shoot proliferation as well as elongation was very poor. Increase in concentration of sucrose favoured shoot development with a maximum of 5.4 shoots per explant at 3%. Increased concentrations of sucrose concomitantly increased the number of necrotic shoots (Table 5.6).

Although sucrose is the most widely used carbohydrate and carbon source in tissue culture, some reports indicate that it may cause hypoxia and ethanol accumulation due to fast metabolism. The addition of carbon source could also bring a significant decrease in the media osmotic potential (NETO and OTONI, 2003 and references therein). These conditions could in turn interfere with the nutrient uptake process. This interference would most likely result in the failure of absorption or diffusion of some important elements. Increasing sucrose concentration increased the incidence of STN and improved shoot multiplication. Since the ultimate goal of any tissue culture system is to have an improved multiplication rate of healthy plants, an alternative solution to overcome STN must be found.

To study the effect of different sugars (two monosaccharides: glucose and fructose; two disaccharides: maltose and sucrose) on shoot proliferation and necrosis, equi-molar (0.086 M) concentration of different sugars were tried. Fructose completely inhibited the development as well as elongation of shoots. On glucose containing medium, abnormal shoots were produced, they were thick and stunted. Supplementation of maltose to the culture medium resulted in the development of maximum number of necrotic shoots (Table 5.7).

Table 5.4: Effect of *m*T and *m*TR with and without IAA on growth and STN of *H. procumbens.* NNoST = number of normal shoot-tips; NNeST = number of necrotic shoot-tips

Treatment (µM)	Shoot length (cm)	No. of branches	NNoST	NNeST	Fresh weight (g)	Necrotic shoots (%)
Control	3.03±0.24^{ab}	1.5±0.16^{c}	1.5±0.16^{bc}	0 ^{c}	0.14±0.02^{cd}	0
5 *m*T	4.05±0.16^{ab}	5.9±0.84^{a}	3.8±0.63^{a}	2.1±0.46^{b}	0.99±0.22^{bc}	35.59
5 *m*TR	4.00±0.27^{ab}	4.4±0.43^{b}	3.3±0.32^{a}	1.1±0.22^{bc}	0.56±0.07^{c}	25
5 BA	4.81±0.35^{a}	4.3±0.47^{b}	2.9±0.48^{ab}	1.4±0.29^{bc}	1.69±0.32^{ab}	32.56
5 BA + IAA	3.43±0.17^{ab}	6.2±0.66^{a}	2.4±0.62^{ab}	3.8±0.56^{a}	2.26±0.13^{a}	61.29
5 *m*T + IAA	3.75±0.14^{ab}	6.9±0.44^{a}	2.6±0.57^{ab}	4.3±0.58^{a}	1.39±0.15^{b}	62.32
5 *m*TR+ IAA	3.60±0.43^{ab}	5.3±0.43^{ab}	3.3±0.58^{a}	2±0.37^{b}	1.31±0.09^{b}	37.73
10 BA + IAA	6.78±2.9^{a}	4.2±0.46^{b}	2.1±0.36^{ab}	2.1±0.49^{b}	2.03±0.18^{a}	50
10 *m*T + IAA	3.35±0.16^{ab}	7.2±0.59^{a}	4.3±0.39^{a}	2.9±0.74^{ab}	2.1±0.19^{a}	40.28
10 *m*TR + IAA	2.90±0.22^{ab}	6.9±0.48^{a}	4.6±0.55^{a}	2.3±0.37^{b}	2.45±0.36^{a}	33.33

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

Table 5.5: Effect of sub-culturing on growth and STN of *H. procumbens*

Treatment	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
Continuous for 4 weeks	4.92±0.13[b]	28[a]	5.09±0.09[b]
Sub culturing after 2 weeks	6.84±0.22[a]	19[b]	6.97±0.08[a]

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

Table 5.6: Effect of sucrose concentration on growth and STN of *H. procumbens*

Sucrose treatment	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
1%	2.16±0.11[c]	18[b]	2.45±0.02[c]
2%	4.36±0.13[b]	21[ab]	3.88±0.07[b]
3%	5.36±0.09[a]	25[a]	4.54±0.05[a]
4%	5.10±0.15[a]	26[a]	4.64±0.06[a]

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

Table 5.7: Effect of sugars on growth and STN of *H. procumbens*

Sugars (0.086 M)	Average number of shoots/explant	Necrotic shoots (%)	Average shoot length (cm)
Sucrose	6.01±0.17[a]	29[d]	6.08[a]
Glucose	1.82±0.11[b]	37[c]	3.93[b]
Maltose	1.52±0.13[b]	76[a]	2.65[c]
Fructose	0.59±0.15[c]	64[b]	*

* shoot buds failed to attain measurable length

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

5.3.6 Effect of Ca and B on STN

Increasing the concentration of Ca and B separately reduced the incidence of STN. Simultaneous addition of higher levels of these ions however, had a detrimental effect on growth. Although high concentrations of Ca reduced STN without affecting growth and regeneration, an elevated B concentration was toxic to the plant and inhibited growth considerably. There are a number of reports that indicate that increasing the concentration of Ca and/or B in the culture medium reduces the problem of STN (VIEITEZ *et al.*, 1989; KATAEVA *et al*., 1991; BARGHCHI and ALDERSON, 1996; PIAGNANI *et al*., 1996).

Other reports indicate that Ca is involved in cellular growth and differentiation, enzymatic activity and membrane permeability (see HIRSCHI, 2004 and HEPLER, 2005 for reviews). Thus Ca deficiency could result in the disturbance of metabolic activities of growing tissues; which in turn results in growth abnormality such as STN. ABDULNOUR *et al*., (2000) studied the effect of B on Ca uptake in micropropagated potatoes and found that excess B can adversely affect Ca uptake. Medium with a high B level decreased Ca content in shoots and leaves but medium with B content four times less than MS enhanced Ca uptake. This could therefore, provide an explanation for our results where increasing the concentration of both B and Ca had an inhibitory effect on the growth of *H. procumbens* plantlets.

Another explanation for the effect of Ca in STN would be its mode of transport in the plant system. HIRSCHI (2004) in his review stated that Ca in the xylem sap needs a transpiration system for upward transport and translocation and indicated the immobile nature of this ion once deposited. The apparent lack of an efficient transpiration stream in culture vessels and in explants (see section 5.3.7) coupled with the limited mobility of this ion could therefore be the causes for the deficiency symptoms observed in the form of STN in young tissues. The fact that STN was observed in cultures with the highest concentration of Ca (up to 15 mM, five times more than the normal full strength MS medium), indicated that the problem of

transport of this ion could have contributed more to STN than its quantity in the medium.

Table 5.8: The effect of calcium and boron concentration on growth and STN of *H. procumbens*.

Treatments (mM)	Average shoot length (cm)	Fresh weight (g)	Dry weight (g)	Mean No. of necrotic shoots per jar
Control*	5.26±0.28^{a}	6.98±0.26^{ab}	0.23±0.02^{ab}	4.80±2.60^{b}
6 Ca	4.36±0.31^{ab}	6.35±0.70^{abc}	0.23±0.01^{ab}	1.60±0.67^{bcde}
9 Ca	4.97±0.25^{a}	5.89±1.33^{abc}	0.20±0.06^{ab}	3.20±1.35^{bcde}
12 Ca	4.27±0.30^{ab}	7.57±1.17^{a}	0.33±0.06^{a}	1.40±0.93^{bcde}
15 Ca	4.70±0.37^{ab}	5.98±1.21^{abc}	0.18±0.05^{ab}	1.00±0.63^{cde}
0.2 B	4.58±0.41^{ab}	7.43±0.82^{a}	.032±0.03^{a}	4.40±0.93^{bc}
0.3 B	4.37±0.27^{ab}	7.16±1.42^{ab}	0.30±0.06^{ab}	3.20±0.92^{bcde}
0.4 B	4.08±0.53^{ab}	4.81±1.26^{abc}	0.24±0.06^{ab}	0.40±0.40^{de}
0.5 B	3.68±0.36^{b}	3.69±0.84^{c}	0.16±0.03^{b}	0.00±0.00^{e}
6 Ca + 0.2 B	4.47±0.28^{ab}	4.81±1.21^{abc}	0.25±0.04^{ab}	8.00±1.14^{a}
9 Ca + 0.3 B	3.68±0.45^{b}	4.04±0.64^{bc}	0.24±0.04^{ab}	4.00±1.41^{bcd}
12 Ca + 0.4 B	3.50±0.28^{b}	3.46±0.67^{c}	0.22±0.03^{ab}	1.20±0.37^{bcde}
15 Ca + 0.5 B	3.63±0.51^{b}	4.31±0.61^{abc}	0.24±0.03^{ab}	1.80±0.49^{bcde}

* The control plants were cultured in half-strength MS media.

Values represent mean ± standard error. Values followed by the same letter in each column are not significantly different ($p \leq 0.05$).

Increasing the amount of B in the medium to more than 200 µM significantly reduced STN but it also reduced shoot multiplication (BARGHCHI and ALDERSON, 1996). This result is in agreement with our findings. These authors further indicated that B deficiency in vascular plants (characterized by cessation of cell division in the apical meristem) could lead to a number of secondary effects such as perturbation of auxin metabolism, increased lignification and phenol accumulation (BARGHCHI and ALDERSON, 1996 and references therein).

5.3.7 Aeration and rooting effects

Throughout this study it was observed that cultures in culture tubes sealed with Parafilm were more prone to STN, excessive branching (reduced elongation) and hyperhydricity as opposed to those cultures in loosely closed screw-cap jars where plants were much healthier. SHA *et al*., (1985) also reported similar results in 'Norland' shoot cultures (*Solanum spp*) with this symptom being particularly evident in cultures with low Ca levels. They also observed that plants grown in Parafilm-sealed vessels had reduced weight and increased branching. It has long been known that aeration plays a considerable role in plant growth and development via its effect on the transpiration stream. Transpiration stream on the other hand plays a pivotal role in the translocation of mineral nutrients through the xylem. In an experiment on *Dianthus* microplants, CASSELLS and WALSH (1994) demonstrated that allowing high moisture vapour loss from culture vessels increased microplant establishment and calcium accumulation in the leaves and enhanced stomatal functioning, presumably by increasing transpiration *in vitro*. Therefore, increased rates of transpiration could most likely result in an increased rate of nutrient flow to the actively growing meristematic regions (BIDDULPH *et al*., 1961; KOHL and OERTLI, 1961; BOWEN, 1972; BARGHCHI and ALDERSON, 1996). Thus a reduced transpiration stream means reduced nutrient flow to growing shoots, resulting in the deficiency of some mineral nutrients. This problem is particularly serious in tissue culture systems where culture vessels are characterized by a very humid atmosphere which suppresses the transpiration stream of cultured shoots. In such cases other mechanisms such as root pressure become limited means for plantlets to absorb nutrients from the culture medium. In the present study, rooted plantlets of *H. procumbens* in a rooting medium slowly overcome the problem of STN. This effect was particularly pronounced with plants treated with *m*T and *m*TR while BA-treated plants failed to show a similar effect. BARGHCHI and ALDERSON (1985) reported that STN did not occur in rooted shoots of *Pistacia vera* L.; a result identical to our findings. MNG'OMBA *et al*., (2007) also published a similar finding for jacket plum.

Our findings suggest that plants in a multiplication stage (having no or only few roots) could suffer from mineral deficiency due to a reduced transpiration stream and absence of root pressure. The problem becomes particularly serious in the case of less mobile minerals such as Ca and B (RAVEN, 1977) where the demand by the plantlets exceeds the supply from the medium. Although this problem can partly be alleviated by improving aeration in the culture vessels as was noted in *H. procumbens* cultures, it should be done carefully as excessive reduction of vessel humidity can result in reduction in, or total inhibition of, shoot multiplication as a result of tissue desiccation due to increased evaporation. Excessive aeration could also compromise the sterility of the culture environment by giving access to pathogens (SHA *et al*., 1985).

5.3.8 Rooting and acclimatization of tissue cultured *H. procumbens*

The initial attempt to root the plantlets produced 100% rooting in just 10 days after transfer to rooting media (Figure 5.1 D). An interesting observation was that plantlets with necrotic symptoms to the rooting media were capable of reverting it or at least, their growth and rooting *in vitro* was not affected. This observation is contrary to some reports which suggest that the possible cause of STN is the omission of cytokinins from the rooting media to counteract their anti-rooting activity (VIEITEZ *et al.*, 1989; KATAEVA *et al*., 1991; PIAGNANI *et al*., 1996). These reports explain that the omission or reducing to very low level of cytokinin leads to depletion of cytokinin from the shoots resulting in shoot degeneration due to failed cell division. Results of this study therefore, suggest that STN in *H. procumbens* is a more complex phenomenon controlled by multiple factors. The fact that STN increased with an increase in cytokinin concentration also indicated that cytokinins play a negative role in the process. There are consistent reports indicating that inclusion of auxin in culture media aggravates STN (see section 2.3). In this study plantlets cultured in rooting media containing IAA alone did not suffer from STN; suggesting that the effect of the different media components on STN could be species dependent.

When transferring rooted plantlets to pots, it was found that roots were very weakly attached to the plant and easily fell off. It was therefore, imperative to carefully separate the plants from the agar media with at least some of their roots attached. This problem led to the idea of using liquid rooting media. This was tried but resulted in serious problem of hyperhydricity.

After carefully removing plants from the rooting media using water, plants were potted in a 1:1 ratio of sand and soil. All of the plants kept in the mist house were dead within one week after transfer. Plants transferred directly to the greenhouse, with daily light watering for the first week, once every three days for the second week and a watering frequency of once a week thereafter had a survival rate of more than 70%. After six weeks growth, acclimatized plants were re-potted to bigger pots for tuber formation. These plants were fully established, grew vigorously and produced flowers and tubers (Figure 5.1 E). The tubers were kept for a comparative pharmacological study for the presence of the bioactive compounds in comparison with tubers from the natural habitat.

6. Micropropagation and somaclonal variation in banana

6.1 Introduction

Bananas and plantains (*Musa spp*. L.) are giant perennial herbs widely cultivated in the humid and sub-humid tropics as main source of carbohydrate. The present day bananas are believed to be the evolution products of intra- and inter-specific hybrids of the two diploid wild species, *Musa acuminata* Colla. and *Musa balbisiana* Colla., in the EuMusa series of the genus *Musa* of the family Musaceae (VUYLSTEKE, 1998 and references therein).

For centuries, conventional propagation of bananas was done using suckers. Increased population growth and the spread of some serious diseases and pests of this crop, necessitated the need for modern propagation techniques. In addition to the obvious reasons for increasing productivity and uniformity, VUYLSTEKE (1998) outlined the following reasons for the importance of banana micropropagation: -

- Problems in germplasm exchange due to the lengthy quarantine period required to clear conventional propagules;
- The risk of disseminating disease and pests which affects the quality of germplasm conservation in the field;
- The bulkiness of conventional propagules and poor phytosanitary quality compromises distribution of selected planting material; and
- Complexity of genetic improvement by conventional hybridization due to low seed fertility and the trisomic pattern of gene inheritance.

The use of shoot-tip culture for the propagation of banana is well established. The first banana tissue culture report appeared in 1972 from Taiwan (MA and SHII, 1972 cited in VUYLSTEKE, 1998). The past three decades thereafter, resulted in a tremendous increase of research both in the development and improvement of this modern technique. The low multiplication rate and the occurrence of a high level of

somaclonal variation limited the widespread use of this method (for details and references see section 2.4).

Banana micropropagation has long been optimized (see VUYLSTEKE, 1998 for details). This section of the research project will therefore, focus only on assessing the potential of topolins as alternatives to BA in banana tissue culture. This attempt results from some recent promising results on the role of toplins in tissue culture. The ability of *m*T to improve acclimatization and *ex vitro* survival rate in *Spathiphylum* spp. (WERBROUCK *et al*., 1996), *m*TR improving survival rate of potato cultures (BAROJA-FERNANDEZ *et al*., 2002), the histogenic stability and anti-senescent effects observed by *m*T derivatives on petunia and rose cultures respectively (BOGAERT *et al.*, 2006) are some examples of these promising results.

6.2 Material and methods

6.2.1 Micropropagation

Tissue cultured ‘Williams’ and ‘Grand Naine’ bananas at the third multiplication cycle were purchased from African Biotechnologies Pty. Ltd., South Africa. Explants were prepared according to the protocol outlined by KRIKORIAN and CRONAUER (1984) with some modification. Aseptically established *in vitro* plantlets were cut transversely to separate leaves and produce a section of pseudostem approximately 1 cm in length, including an intact vegetative bud. The lower part of the pseudostem was trimmed to remove darkened or necrotic tissues and the sheath removed carefully by peeling. The explants were then cut in half longitudinally. These explants were then cultured in a screw-cap jar containing 50 ml of modified MURASHIGE and SKOOG (1962) macro and microelements (VUYLSTEKE, 1998), supplemented with sodium dihydrogen orthophosphate (0.38 g l^{-1}), ascorbic acid (0.18 g l^{-1}), adenine sulphate (0.2 g l^{-1}), sucrose (30 g l^{-1}), Gelrite (2 g l^{-1}), and growth the regulators auxin; 1 mg l^{-1} and cytokinins; BA, Me*m*T, Me*m*TR, *m*T and *m*TR at 7.5, 15 and 30 µM concentration. After adjusting the pH to 5.8 the media were autoclaved at 121^{o}C and

103 kpa for 20 min. Filter sterilized ascorbic acid was then added when the media were just above the solidification temperature (about 50^{o}C). Cultures were incubated in a growth room having 16 h light/8 h dark conditions with an irradiance of 43 µmol m^{-2} s^{-1} at a temperature of 26±1^{o}C. There were five replicates per treatment each having three explants. Sub-cultures were made at six-week intervals. After initial observations of growth responses, BA, *m*T and *m*TR were compared for multiplication efficiency at 22.2 µM (other components were kept constant) (VUYLSTEKE, 1998). Data on growth parameter were analyzed using either GenStat 9th edition or the SPSS 15.0 version. Abnormality index was calculated by taking the ratio of abnormal to normal shoots. The lower the abnormality index values the better the treatments are.

6.2.2 Analysis of somaclonal variation

Sufficient leaf samples from each of BA, *m*T and *m*TR-treated 'Williams' cultivar were collected at the 7th multiplication cycle. The control samples were collected from the initial plantlets at the 3rd multiplication cycle before being subjected to any treatment. Genomic DNA was extracted and quantified using protocols described by RICHARDS (1997) and (HILLS and VAN STADEN, 2002) with slight modifications (see Appendix 2 a-c for details). DNA (2 µg) was diluted in 300 µl of TE buffer for all DNA samples to make a final uniform concentration of 0.006 µg μl^{-1} of DNA. Diluted DNA template was then used to add 54 ng of DNA to the 25 µl of PCR reaction mixture (BAIRU *et al*, 2006).

DNA amplification reactions were performed in volumes of 25 µl containing reaction mixture as described by DAMASCO *et al*. (1996b) with slight modifications. These included 2.5 µl PCR Manufacturer's Reaction Buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg ml^{-1} gelatine, pH 8.3; Roche Diagnostics GmbH, Mannheim, Germany), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 µM of OPC-15 random decamer primer (Operon Technologies, Alameda, CA, USA), 54 ng of banana genomic DNA and 1.5 units of

Taq DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Sterile HPLC grade water was used to adjust the final volume to 25 µl and overlaid with 50 µl of paraffin oil. Amplification was performed in a Hybaid Thermal Reactor (HYBAID Ltd., UK, HYBAID 1991 Model). The amplification reaction was performed at an initial denaturation temperature of 95^{o}C for 1 min, 36^{o}C for 20 sec and 72^{o}C 2 min (1 cycle) followed by 45 cycles of each 95^{o}C for 10 sec, 36^{o}C for 20 sec and 72^{o}C for 2 min with a final elongation step (1 cycle) of 72^{o}C for 5 min and 35^{o}C for 1 min.

The amplification products were analysed by gel electrophoresis in 1.5% agarose (HISPANAGAR, Burgos, Spain) containing 0.25 µg ml^{-1} of ethidium bromide. After thermocycling, 3 µl of gel-loading buffer were mixed with the 25 µl reaction mixture, and 15 µl were loaded onto a 1.5% agarose gel. The gel was run in 1 x TAE (running buffer) at 50V (5 V/cm) for 3.5 h (HOEFER SCIENTIFIC INSTRUMENTS, USA). DNA molecular weight marker XIV (100 bp) (Roche Diagnostics GmbH, Mannheim, Germany) was included on each gel. Visualization of the amplification product was made under UV light, 6x8 W – 312 nm tube, (UVItec Limited, Cambridge, UK , BTS-20. M, model). The image was then saved for statistical analysis.

The analysis was done using three replicates, having 20 samples each, to give a total of 60 samples per treatment. The analysis was made based on the presence of the dwarf off-type due to the high prevalence of this variant (SMITH and DREW, 1990b; RAMAGE *et al*., 2004) in the tissue culture of ‘Williams’ cultivar (*Musa* spp. AAA, sub group Cavendish) and the absence of other known markers to detect other variants. The amplification products (the gel images) of each sample were then scored for the presence or absence of the dwarf specific marker OPC-15_{1500} (BAIRU *et al*., 2006) using the molecular weight marker. An example of PCR products separated by gel electrophoresis is presented in Figure 6.1. Percentage variation from each replicates was then Arc-sign transformed and analyzed for significance using the MINITAB package.

6.3 Results and discussion

6.3.1 Micropropagation

Results of the micropropagation experiment revealed that *m*T produced the largest number of shoots followed by *m*TR (Tables 6.1 and 6.2). An higher gross multiplication rate (total number of shoots divided by initial number of explants) was obtained from the *m*T and *m*TR treatments at 30 µM concentration for 'Williams' and at 15 µM for 'Grand Naine'. The high abnormality index recorded at 30 µM for all the treatments makes this treatment unusable. The 6.13 shoots per explant obtained for 'Williams' at 15 µM *m*T was in fact comparable to the 6.4 shoot per explant at 30 µM *m*T (Table 6.1). Shoot clusters produced by Me*m*T and Me*m*TR were very small in size compared to BA, *m*T and *m*TR treatments for both cultivars. The topolin treatments inhibited rooting at all concentrations compared to BA and the control.

Previous reports (CRONAUER and KRIKORIAN, 1984; JARRET *et al*., 1985; VUYLSTEKE and DE LANGHE, 1985) indicated that 5 mg l^{-1} (22.2 µM) is the optimum cytokinin concentration for most banana tissue cultures. The shoot multiplication and rooting effect of BA, mT and *m*TR at this concentration was therefore, examined. Results indicated that *m*TR produced a significantly larger number of shoots (5.3 normal shoots per initial explant) compared to BA. *m*T was also superior to BA but the difference was not statistically significant (Table 6.3). These results were consistent with those presented in Tables 6.1 and 6.2 although the total number of normal shoots was higher (5.3) at the 22.2 µM concentration of *m*TR as opposed to 4.9 and 2.8 respectively of 15 and 30 µM *m*TR. A similar trend was observed for *m*T treatment. The optimum concentration (22.2 µM) reported by many authors also applies for the topolins tested in this study. It could, therefore, be concluded that the topolins tested can be used as alternatives to BA due to their better shoot multiplication effect at equi-molar concentrations.

Table 6.1: The role of topolins in the micropropagation and incidence of abnormality of 'Williams' banana. TNS = total number of shoots; NNS = number of normal shoots; NAS = number of abnormal shoots; Abnormality index = the ratio of abnormal to normal shoots and MR = multiplication rate

Treatments	TNS per jar	NNS per jar	NAS per jar	Abnormality index	MR
Control	5.6±0.51^{f}	5.2±0.37^{e}	0.4±0.24^{e}	0.07±0.04^{d}	1.87±0.17^{e}
7.5 µM BA	9.6±1.53^{ef}	7.4±1.21^{cde}	2.2±0.66^{cde}	0.32±0.13^{bcd}	3.20±0.51^{ef}
7.5 µM Me*m*T	14.0±1.09^{bcde}	11.6±1.16^{abc}	2.4±0.40^{cde}	0.22±0.05^{cd}	4.67±0.36^{bcde}
7.5 µM Me*m*TR	11.4±0.67^{de}	9.8±0.58b^{cde}	1.6±0.40^{e}	0.17±0.04^{cd}	3.80±0.22^{de}
7.5 µM *m*T	14.2±2.11^{bcde}	12.4±1.91^{ab}	1.8±0.37^{de}	0.15±0.02^{cd}	4.73±0.70^{bcde}
7.5 µM *m*TR	11.8±1.32^{de}	11.4±1.08^{abc}	0.4±0.24^{e}	0.03±0.02^{cd}	3.93±0.44^{de}
15 µM BA	12.2±1.24^{cde}	6.0±0.45^{de}	6.2±1.11^{ab}	1.05±0.18^{ab}	4.07±0.41^{cde}
15 µM Me*m*T	15.0±1.48^{abcd}	9.6±0.40^{bcde}	5.4±1.21^{abc}	0.55±0.12^{abcd}	5.00±0.49^{abcd}
15 µM Me*m*TR	17.0±1.55^{abc}	9.6±0.51b^{cde}	7.4±1.63^{a}	0.79±0.18^{abcd}	5.67±0.51^{abc}
15 µM *m*T	18.4±2.16^{ab}	10.2±2.03^{abcd}	7.8±2.50^{a}	1.09±0.58^{ab}	6.13±0.72^{ab}
15 µM *m*TR	18.0±1.30^{ab}	14.8±1.59^{a}	3.2±0.53^{bcde}	0.23±0.05^{cd}	6.00±0.43^{ab}
30 µM BA	11.8±0.97^{de}	5.4±0.67^{e}	6.4±1.29^{ab}	1.32±0.32^{a}	3.93±0.32^{de}
30 µM Me*m*T	16.0±2.34^{abcd}	10.6±2.96^{abcd}	5.4±1.33^{abc}	0.86±0.41^{abc}	5.33±0.78^{abcd}
30 µM Me*m*TR	12.8±1.46^{cde}	6.6±1.03^{de}	6.2±1.16^{ab}	1.07±0.29^{ab}	4.27±0.48^{bcde}
30 µM *m*T	19.8±1.80^{a}	12.4±1.86^{ab}	6.8±1.28^{ab}	0.65±0.20^{abcd}	6.40±0.60^{a}
30 µM *m*TR	13.8±1.83^{bcde}	8.6±1.96^{bcde}	5.2±0.49^{abcd}	0.89±0.37^{abc}	4.60±0.61^{bcde}

Table 6.2: The role of topolins in the micropropagation and incidence of abnormality of 'Grand Naine' banana. TNS = total number of shoots; NNS = number of normal shoots; NAS = number of abnormal shoots; abnormality index = the ratio of abnormal to normal shoots and MR = multiplication rate

Treatments	TNS per jar	NNS per jar	NAS per jar	Abnormality index	Gross MR
Control	5.60±0.51^{g}	5.20±0.37^{f}	0.40±0.24^{i}	0.07±0.04^{d}	1.87±0.17^{g}
7.5 µM BA	11.00±0.71^{def}	6.00±0.00^{ef}	5.00±0.71^{cdefg}	0.83±0.12^{bcd}	3.67±0.24^{def}
7.5 µM Me*m*T	9.60±0.75^{defg}	6.40±0.51^{def}	3.20±0.80^{fghi}	0.53±0.16^{cd}	3.20±0.25^{defg}
7.5 µM Me*m*TR	10.00±1.14^{defg}	7.60±0.51^{cdef}	2.40±0.75^{ghi}	0.30±0.09^{cd}	3.33±0.38^{defg}
7.5 µM *m*T	9.20±1.24^{efg}	7.60±0.93^{cdef}	1.60±0.51^{hi}	0.21±0.06^{cd}	3.07±0.41^{efg}
7.5 µM *m*TR	8.60±1.35^{fg}	8.00±1.58^{bcdef}	0.60±0.40^{i}	0.11±0.08^{cd}	2.87±0.45^{fg}
15 µM BA	14.20±2.40^{cde}	9.80±2.18^{abcde}	4.40±0.75^{efgh}	0.59±0.23^{cd}	4.73±0.80^{cde}
15 µM Me*m*T	13.60±1.94^{cdef}	10.40±1.21^{abcd}	3.20±0.73^{fghi}	0.47±0.26^{cd}	4.53±0.64^{cdef}
15 µM Me*m*TR	14.60±1.08^{bcd}	11.40±1.29^{abc}	3.20±1.24^{fghi}	0.35±0.17^{cd}	4.87±0.36^{bcd}
15 µM *m*T	20.60±1.25^{ab}	13.40±0.98^{a}	7.20±0.80^{abcde}	0.55±0.07^{cd}	6.87±0.42^{a}
15 µM *m*TR	18.60±1.69^{abc}	13.80±1.07^{a}	4.80±0.73^{defg}	0.34±0.04^{cd}	6.20±0.56^{abc}
30 µM BA	11.60±1.94^{def}	4.20±1.07^{f}	7.40±1.29^{abcd}	2.40±0.73^{a}	3.87±0.65^{def}
30 µM Me*m*T	12.60±2.44^{def}	4.20±1.56^{f}	8.40±1.50^{ab}	1.51±0.48^{b}	4.20±0.81^{def}
30 µM Me*m*TR	14.00±1.58^{cde}	6.00±1.09^{ef}	7.80±1.20^{abc}	1.47±0.31^{b}	4.67±0.53^{cde}
30 µM *m*T	19.40±2.64^{a}	10.00±1.55^{abcde}	9.40±1.50^{a}	0.97±0.13^{bc}	6.47±0.88^{ab}
30 µM *m*TR	18.00±0.45^{abc}	11.80±1.02^{ab}	6.20±0.58^{bcdef}	0.56±0.10^{cd}	6.00±0.15^{abc}

Table 6.3: The effect of different cytokinins on banana micropropagation

Treatments	22.2 µM		
	No. of normal shoots/explant	No. of roots	Average shoot length (cm)
Control	1.5[b]	11.8[a]	7.85
BA	3.0[ab]	10.4[a]	5.25
*m*T	4.8[a]	3.3[b]	6.20
*m*TR	5.3[a]	0.4[c]	6.45
SE	**0.909**	**1.473**	**0.942**
F prob.	**0.001**	**0.001**	**0.072**
LSD	**1.866**	**3.022**	**Ns***

*ns - indicates non significant treatment effects

BA was the most toxic cytokinin based on the higher abnormality index recorded for both cultivars (Tables 6.1 and 6.2). The inhibitory and/or toxic effects observed at higher concentrations are not new to banana tissue culture. Although concentrations as high as 10 mg l^{-1} were used (DORE SWAMY *et al*., 1983), higher concentrations generally inhibited shoot multiplication (WONG, 1986; VUYLSTEKE, 1998 and references therein). 'Williams' banana was more sensitive than 'Grand Naine' to higher cytokinin concentrations. It is well documented that bananas show a wide range of dose dependent responses among and within the genomic groups of *EuMusa* series. VUYLSTEKE (1998) suggested that differences in sensitivity could be due to cultivar dependent responses to the different cytokinin concentrations.

In contrast to previous findings with topolins (WERBROUCK *et al*., 1996; chapter four of this thesis), *m*T and *m*TR had an inhibitory effect on rooting when compared to BA and the controls (Table 6.3). Particularly striking is the near complete inhibition of rooting by *m*TR, a compound that promoted rooting of *Aloe polyphylla* shoots at concentrations where both BA and *m*T completely inhibited the process. In his banana shoot-tip culture manual, VUYLSTEKE (1998) emphasized the importance of a high cytokinin-auxin ratio for efficient shoot multiplication. The author also indicated that best multiplication was achieved

without associated rooting. Therefore, based on the existing knowledge the following assumptions may explain the results obtained: -

1. The physiological effects of topolins on root formation could be species dependent;
2. In the presence of a constant auxin concentration, the difference in the molecular weight of the cytokinins could have significantly influenced the cytokinin-auxin ratio to the extent of inhibition of rooting. For example 22.2 µM BA equals 5 mg/L whereas 22.2 µM *m*TR equals 8.3 mg/L; and
3. There appears to be an inverse correlation between shoot multiplication and root formation.

6.3.2 Analysis of somaclonal variation

Results of the analysis of somaclonal variation (Table 6.4) revealed that there was no significant difference between treatments. Nevertheless all the treatments were significantly different from the control (plants from the 3rd multiplication cycle). There was a significant increase in variation rate between the 3rd and 7th multiplication cycles. Results of the amplification product revealed the presence of the polymorphic 1.6 kb RAPD marker previously reported using OPC-15 primer (BAIRU *et al*., 2006) and OPJ-04 primer (DAMASCO *et al*., 1996b). The present result is therefore additional proof of the reliability of the dwarf specific marker. The fact that previous authors reported this variant from different banana cultivars could suggest the presence of only one type of dwarf off-type.

Table 6.4: The effect of *m*T and *m*TR on somaclonal variation of 'Williams' banana

Treatments	No. of samples analyzed	Variation (%)
Control	60	5^{b}
BA	60	23^{a}
*m*T	60	19^{a}
*m*TR	60	21^{a}

The absence of any statistically significant difference between the treatments could be explained in two ways. Firstly, all the cytokinins tested at equi-molar

concentration (22.2 µM) do not have an effect on somaclonal variation in which case the variation observed is the result of factors known to affect somaclonal variation such as number of subcultures. Secondly, it could be due to the carry-over effect of BA, since the cultures were initiated and maintained in media containing BA for three multiplication cycles (sub-cultures).

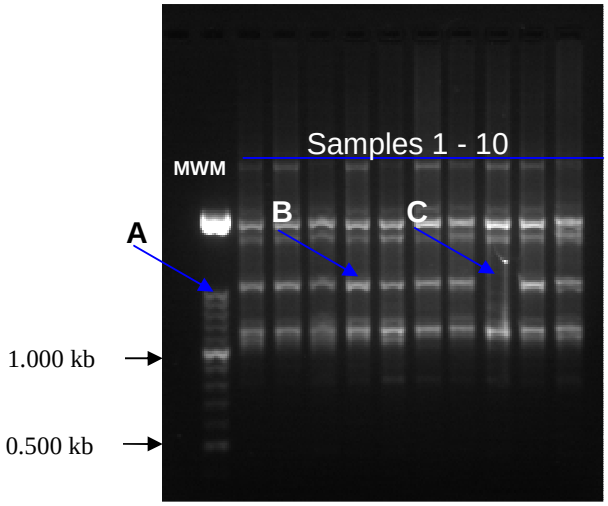


Figure 6.1: An example of amplification products of the control samples separated by agarose gel electrophoresis. A = 1500 bp fragment of the MWM; B = the dwarf specific marker (OPC-15_{1500}) consistently amplified by normal plants but absent in dwarfs; and C = dwarf off-type sample lacking the OPC-15_{1500} fragment found in normal samples

The latter could be of particular importance given the very stable nature of BA and its metabolites in plant tissue and being an apparent cause of heterogeneity (WERBROUCK *et al*., 1995). The possible effect of topolins on somaclonal variation however, should not be overlooked. BOGAERT *et al.* (2006) reported the histogenic stability effect of the topolin Me*m*TR on petunia cultures. Unfortunately this was reported towards the completion of the experimental work of this study so the author opted to only investigate those topolins that showed good potential to act as replacements for BA, *m*T and *m*TR, based on results of shoot multiplication experiments (Table 6.1 and 6.2). It may therefore, be worthwhile to investigate the role of topolins on somaclonal variation using plant materials which have not been in contact with applied BA.

7. Conclusions and recommendations

The results of the soybean callus bioassay are useful additions to the ever growing knowledge of synthesis and use of cytokinins. The results demonstrated the importance of structure activity relationships in cytokinin research. The differential effects of the relative biological activity of different cytokinins in different bioassay system highlight the importance of activity profiling of these cytokinins using the different bioassay systems. With time, this activity profiling should include the entire family of topolins including their corresponding nucleotides, nucleosides, glucosides as well as related derivatives.

The micropropagation protocol developed for *A. polyphylla* has been tested on a larger scale and can be recommended for commercial propagation of this species. The complete control of hyperhydricity using *m*T and the absence of the negative *ex vitro* effect frequently observed with BA-treated cultures warrants related research for species with similar problems. Currently there are ongoing trials to test the protocol on different aloes.

In view of the shoot multiplication potential of *H. procumbens*, all cuttings of nodal segments and the apical shoots were capable of regeneration. It is therefore possible to generate a very large number of plants in a very short period for commercial propagation. Virtually, all of the tissue culture conditions tested affected STN in some way. The optimum protocol developed, following the investigations made in this study contains the results from each experiment. Auxin-free half strength MS media supplemented with 3% sucrose, 3-5 µM *m*TR, 1% agar, 6 mM Ca, 0.1 g l^{-1} myo-inositol, pH 5.8 and a sub-culture period of two weeks are requirements for shoot multiplication. The very complex nature of STN in this species necessitates further investigations on boron transport and the role of sugar alcohols in this process as well as investigating the interaction between Ca, B and PGR's using labelled compounds.

Low multiplication and high somaclonal variation rates are characteristics of the existing banana micropropagation protocol. The higher multiplication rate obtained using topolins is therefore, an important addition to our knowledge for application to banana tissue culture. The inhibitory effect of these cytokinins on rooting of banana cultures indicates that the rooting response is species specific. The physiological effects of topolins on rooting of plants require detailed investigations. The results reported on the effect of topolins on somaclonal variation should not lead to general conclusions mainly due to the nature of the experimental plants used and associated carry-over effects. Recent reports on histogenic stability effects of some topolins also warrant detailed investigations starting right from the culture initiation stage.

The present results demonstrate the potential topolins hold in alleviating and/or controlling some tissue culture-related problems. The positive effect of topolins on hyperhydricity, STN and banana micropropgation are the first of their kind and serve as foundation for future research. This study aimed at understanding factors contributing to the problems investigated and seeking solutions to alleviate or control them. More stringent research using labelled cytokinins could lead to a better understanding of the mode of action and physiological effects of these compounds.

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Appendices

Appendix – 1

Tissue culture media MS stocks and additives

Table A1-1: MS basal medium full strength (MURASHIGE and SKOOG, 1962)

Stocks	Nutrients	Mass (g) per volume of media		Volume used per liter
		500 ml	1000 ml	
1	NH_3NO_3	82.5	165.0	10
2	KNO_3	47.5	95.0	20
3	$CaCl_2.2H_2O$	22.0	44.0	10
4	$MgSO_4.7H_2O$	18.5	37.0	10
5	NaFe EDTA	2.0	4.0	10
6	KH_2PO_4	8.5	17.0	10
7a	H_3BO_4	0.31	0.62	10
	$ZnSO_4.7H_2O$	0.430	0.860	
	KI	0.0415	0.083	
7b	$MnSO_4.4H_2O$	1.115	2.230	10
8	$NaMoO_4.2H_2O$	0.0125	0.025	10
	$CuSO_4.5H_2O$	0.00125	0.0025	
	$CoCl_2.6H_2O$	0.00125	0.0025	
9	Thiamin HCl (B_1 / Aneurine	0.005	0.01	10
	Niacine (Nicotinic Acid)	0.025	0.05	
	Pyridoxine HCl (B_6)	0.025	0.05	
	Glycine	0.1	0.2	

Additives to the tissue culture media used

Component	Concentration
Sucrose	3.0%
Gelrite	0.2 – 0.3 %
Agar	0.8 – 1%
Adenine Sulphate	0.2 g l^{-1}
Sodium-dihydrogen	0.38 g l^{-1}
Ascorbic acid	0.18 g l^{-1}
Myo-inositol	0.1 g l^{-1}

Appendix – 2

a. DNA extraction recipes

CTAB – extraction solution: (2% (w/v) CTAB; 100 mM Tris.Cl pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NACl)

CTAB/NaCl solution: (10% CTAB; 0.7 M NaCl mixed while heating (65^{o}C) and stirring)

CTAB – precipitation solution: (1% w/v CTAB; 50 mM Tris.Cl, pH 8.0; 10 mM EDTA)

High- salt TE buffer: (10 mM Tris.Cl, pH 8.0; 0.1 mM EDTA, pH8.0; 1 M NaCl)

TE buffer: (10 mM Tris.Cl, pH 7.4, 7.5 or 8.0; 1 mM EDTA, pH 8.0)

Chloroform: iso-amyl alcohol (24:1 ratio)

b. DNA extraction protocol

The protocols described by RICHARDS (1997) and (HILLS & VAN STADEN, 2002) with slight modifications were employed to extract total genomic DNA from *in vitro* grown plantlets.

1. Reagents were prepared according to RICHARDS (1997). These reagents include: CTAB extraction solution, CTAB/NaCl solution, CTAB precipitation solution, high salt TE buffer, TE buffer, 80% ethanol and 24:1 Chloroform:Iso-amyl alcohol (see Appendix 2).
2. Ethanol (100%), Iso-propanol, Polyvinylpolypyrolindone (PVPP) insoluble, 2-Mercaptoethanol (2-ME) and liquid nitrogen were used directly.
3. Sterile mortar and pestle, 1.5 ml Eppendorf, pipette tips (1.5 ml and 100 µl) were prepared.
4. CTAB extraction solution (500 µl) heated to 65^{o}C were added to 3% PVPP (0.015 g) in a 1.5 ml Eppendorf tube. 2-ME (10µl) was added to the mixture to give a final concentration of 2% (v/v) immediately before the start of extraction.
5. Leaf tissue obtained from *in vitro* plantlets (0.25 g) was frozen in liquid nitrogen (-196^{o}C) in a mortar and ground to a fine powder.
6. The ground and frozen tissue was then added to a warm (65^{o}C) PVPP/2-ME/ CTAB extraction solution. The mixture was then incubated for 30 min at 65^{o}C in a water bath, with occasional mixing.
7. The homogenate was then mixed with an equal volume of 24:1 Chloroform:Iso-amyl alcohol by inversion followed by centrifugation at 10,000 rpm (7500 x *g*) for 5 min in a microcentrifuge (HERSTELLER SPINTRON Ltd, Wehingen, Germany . Model, Z 160 M) (Figure 3.1). The upper aqueous part was then recovered.
8. CTAB/NaCl solution (1/10 volume at 65^{o}C) were added to the recovered aqueous phase and mixed well by inversion.
9. The mixture was then extracted with an equal volume of Chloroform/Iso-amyl alcohol. After mixing and centrifuging for 5 min at 10,000 rpm (7500 x *g*) the upper aqueous layer was recovered.
10. One volume (1 ml) of 65^{o}C CTAB precipitation solution was added to the recovered aqueous phase. In most cases the precipitate was visible at this stage but in a situation where it was not visible an additional incubation period of 30 min at 65^{o}C was applied.

11. The mixture was centrifuged for 5 min at 2700 rpm (500 x *g*) and the pellet was recovered by removing the supernatant. The pellet was then dissolved and suspended in high-salt TE buffer (2 ml per gram of starting material).
12. The DNA was subsequently precipitated by adding 0.6 volumes (1.2 mlg^{-1} of starting material) of ice-cold Iso-propanol followed by mixing and centrifuging for 15 min at 10,000 rpm (7500 x *g*).
13. The supernatant was decanted and the pellet first washed with 80% ethanol followed by 100% ethanol. The dried pellet was then suspended in a minimal volume of TE buffer (0.2 ml per gram of starting material). The DNA was then stored at –20^{o}C until further use.

c. DNA quantification

Quantification of DNA was done for each sample using spectrophotometry at A_{260}, A_{280} and A_{320} nm absorbance using a 250 x dilution (4 µl of DNA stock and 996 µl of TE buffer as blank). Ratio, purity and concentration were then calculated using the following formulae:

Ratio = A_{260}/A_{280}

Purity (%) = Ratio/1.8 X 100

Concentration in (µg μl^{-1}) = (Corrected A_{260} X ΔE X dilution)/1000 Where:

corrected $A_{260} = A_{260} – A_{320}$

ΔE = 50 (extinction coefficient for genomic DNA); and dilution = 250 x

d. Theoretical backgrounds of PCR requirements (BAIRU, 2004)

1. Description and requirements of the PCR and its components

According to RYBICKI (2001) the following factors affect the effectiveness of PCR conditions; denaturing temperature and time, annealing temperature and primer design, primer length, degenerate primer, elongation temperature and time, reaction buffer and cycle number. PEAKALL (1997) defined the basic PCR procedures in three major approaches and stated that the choice of an appropriate method depends on the aim of the study. These approaches include;

1. Sequence-Tagged- Site (STS) PCR;
2. Arbitrary-Primed (AP) PCR; and
3. A combination of the two approaches (AFLP)

1. STS – PCR

This procedure uses two different specific primers, complimentary to opposite strand of conserved DNA, to amplify the intervening sequence. Micro-satellite or Simple Sequence Repeats (SSRs) are the best example of genetic markers revealed by STS-PCR (PEAKALL, 1997). The prominent advantages of STS-PCR include;

- Produce a co-dominant single locus genetic marker; as a result the DNA profiles are easier to score than multi loci profile and heterozygotes are readily apparent; and
- In the presence of a successful reaction a positive result is always achieved except in the case of null alleles.

The production of false polymorphism during partial failure of the reaction is the limitation of this system, unlike the multi loci method. The importance of high-resolution electrophoresis is another technical complication of SSRs, specially for dinucleotide repeat SSRs where alleles may differ by only two base pairs (PEAKALL, 1997).

2. AP-PCR (RAPDs)

This procedure involves the use of a single short primer of arbitrary (but known) sequence. Under low stringency PCR conditions, multi loci DNA profiles could be produced with polymorphisms revealed as band presence or absence (WILLIAMS *et al.,* 1990; WELSH and McCLELLAND, 1990). RAPD (Random amplified polymorphic DNA) (WILLIAMS *et al*., 1990), AP-PCR (arbitrary-primed PCR) (WELSH and McCLELLAND, 1990), DAF (DNA amplification fingerprinting) (CAETANO-ANOLLES *et al*., 1991) and ssRAPDs (silver stained RAPDs) (HUFF and BARA, 1993) are acronyms for AP-PCR techniques. The term RAPDs, however, is widely used as a generic term for AP-PCR (PEAKALL, 1997).

Table A2-1: Differences in electrophoresis and visualization techniques of AP-PCR procedures

PCR procedure	Electrophoresis	Visualization technique and staining	Reference
RAPD	Agarose gel	Ethidium bromide stain	WILLIAMS *et al*. (1990)
DAF	Polyacrylamide gel	Silver staining	CAETANO-ANOLLES *et al*. (1991)
ssRAPDs	Polyacrylamide gel	Silver staining	CAETANO-ANOLLES *et al*. (1991)
AP-PCR	Polyacrylamide gel	Radioactive labels	WELSH and McCLELLAND (1990)

RAPDs differ from other PCR techniques in primer length, primer to template ratio, the gel matrix used and in the visualization procedure. Its advantage over other genetic markers is that DNA sequence knowledge is not required, making it theoretically applicable to any organism (KARP *et al*., 1996; DAMASCO *et al*., 1996b; PEAKALL, 1997). Although there are some differences in the type of primer and PCR conditions, the major difference among the PCR procedures is the type of electrophoresis and visualization technique of the DNA fragments (Table A2-1)).

Table A2-2: Characteristics of the different MAAP techniques (CAETANO-ANOLLES, 1994)

Characteristics	DAF	AP-PCR	RAPD
Primer length (nt)	5-15	18-32	9-10
Primer concentration (μM)	3-30	1-10	0.3
DNA concentration ($ng\mu l^{-1}$)	0.01-1	0.1-5	1
Primer/template ratio	5-50 000	1-500	<1
Annealing temperature (^{o}C)	10-65	35-50	35-42
Amplification stringency	Low to high	High to low	Low
DNA separation	PAGE	PAGE	Agarose
Visualization	Silver staining	Radiolabeling	EtBr staining
Product resolution	High	Intermediate	Low
Number of products	10-100	3-50	1-10

Note: nt = number of nucleotides; PAGE = polyacrylamide gel electrophoresis

CAETANO-ANOLLES *et al.,* (1995), however, explained that these distinctions are becoming less functional due to the many modifications such as fluorescent labelling and high resolution polyacrylamide electrophoresis of RAPDs on

automated sequences, and the fluorescent labelling and automated capillary electrophoresis of DAF described in the literature.

CAETANO-ANOLLES *et al*., (1992) suggested a new name that encompasses the three techniques (AP-PCR, RAPD and DAF) with an acronym MAAP (multiple arbitrary amplication profiling). CAETANO-ANOLLES (1994) characterised these three MAAP procedures (Table A2-2) to make an important distinction amongst them since the use of the type of electrophoresis and visualization technique alone for distinction is becoming blurred.

Apart from their advantages MAAP procedures (RAPDs, AP-PCR, and DAF) have the following limitations;

- The markers are dominant and heterozygotes cannot be detected, hence provide less genetic information than co-dominant markers such as allozymes or SSRs;
- In the absence of pedigree analysis, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci;
- The presence of a band of apparently identical molecular weight in different individuals cannot be taken as evidence that the two individuals share the same homologous fragment, although this assumption is commonly made; and
- Single bands on the gel can sometimes be comprised of several co-migrating amplification products.

3. AFLP

This is a relatively new addition to the PCR procedures and is termed as amplified fragment length polymorphism (AFLP). It is essentially intermediate between RFLP and PCR. AFLP involves restriction digestion of genomic DNA followed by selective rounds of PCR amplification of the restricted fragments. The amplified products are radioactively or flourescently labelled and separated on sequencing gel. AFLPs appear to be as reproducible as RFLPs, but are technically more demanding and require more DNA (1μg per reaction) than RAPDs. The advantage

of this technique over RAPD marker is that because of their large genome coverage on average they give 100 bands per gel compared with 20 for RAPDs. AFLPs are very good for mapping and fingerprinting and genetic distances can be calculated between genotypes. They do, however, share many of the limitations with RAPDs (KARP *et al*., 1996).

A successful PCR experiment depends on the optimal reaction conditions, the template DNA, and respective primer. There are, of course, parameters that vary according to applications such as magnesium concentration, pH of the reaction buffer, temperature and time of the different cycling steps. In addition the various components (Table A2-3) may increase the specificity and yield of some reactions. Therefore, it is imperative and is generally recommended to optimise the PCR reaction conditions for each new template primer pair.

Table A2-3: Comparison of concentrations of PCR buffers used in this study with concentrations recommended by INNIS and GELFAND (1990).

Buffer	Recommended concentration	Concentration used in this study
Tris-HCl	10-50 mM	10 mM
KCl	Up to 50 mM	50 mM
$MgCl_2$	0.5-2.5 mM	1.5 mM
Primer	0.2-1 μM	0.2 μM
dNTP	50-200 μM	200 μM
Gelatin or BSA	100 μg ml^{-1}	0.1 mg ml^{-1}
Taq DNA pol.	0.5-1 units	1.5 units
Tween-20	0.05-0.1 % v/v	-
Triton X-100	0.05-0.1 % v/v	-

The number, size and intensity of bands in RAPD's is also affected by several other factors, which includes; PCR buffer, dNTP's, Mg^{2+} concentration, cycling parameters, source of Taq DNA polymerase, conditions and concentrations of DNA and primer type and concentration. The limitations to successful PCR could be overcome by maintaining a constant set of defined reaction conditions and consistency of technical applications (RYBICKI, 2001).

General considerations (RYBICKI, 2001):

- Taq could be inhibited by higher concentration (>50 mM) of KCl or NaCl;
- Taq requires free Mg^{2+} and hence allowance should be made for dNTP's, primers and template;
- Primer annealing, Tm of template, product and primer-template associations, product specificity, enzyme activity and fidelity are affected by Mg^{2+};
- Primer concentration should not go above 1 μM unless there is a high degree of degeneracy and 0.2 μM is usually enough for homologous primers; and
- Although long products may require more, nucleotide concentration need not be above 50 μM each.

1.1 Taq DNA polymerase

This enzyme is the recombinant form of the enzyme from the thermophilic eubacterium, *Thermus aquaticus* BM, expressed in *E. coli*. Taq DNA polymerase consists of a single polypeptide chain with a molecular weight of approximately 95 kD. It is highly active 5'-3' DNA polymerase lacking 3'-5' exonuclease activity. This enzyme exhibits its highest activity at a pH of around 9.0 and a temperature around 75^{o}C, though it is stable to prolonged incubation at elevated temperatures of up to 95^{o}C (BROWN, 1995).

1.2 Primer selection

Primers OPC-15 (5'-GACGGATCAG-3') and OPJ-04 (5'-CCGAACACGG-3') were used for the purpose of this study. INNIS and GELFAND (1990) stated that the following points act as the basis for successful primer selection:

1. Primers should be 17-28 bases in length;
2. Base composition should be 50-60% (G+C);

3. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
4. Tms between 55-80^{o}C are preferred;
5. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
6. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesized preferentially to any other product; and
7. Primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided.

Primer length is another important consideration to be noted. The optimum length of a primer depends on its (A+T) content and Tm of its partner. The most important consideration in choosing a primer is that it should be complex enough so that the possibility of annealing to sequences other than the chosen target would be very low. If a primer is too long, it is difficult to avoid or prevent mismatch pairing and non-specific priming even with high annealing temperatures (INNIS and GELFAND, 1990).

1.3 Denaturing temperature and time

Annealing is the specific complimentary association due to hydrogen bonding of single stranded nucleic acids (NA) (RYBICKI, 2001). To form a stable double stranded hybrid, two complimentary sequences would form hydrogen bonds between their complimentary bases (G to C and A to T or U). Nucleic acids would be made single stranded by heating to a point above the melting temperature (Tm) of the double stranded form for the purpose of annealing, followed by a flush cooling to avoid re-annealing of denatured strands. Loss of Taq activity (denaturation) is strongly influenced by time at a given temperature. Reducing the time increases the number of possible cycles, irrespective of the temperature. Increase in denaturation temperature and decrease in time could also work; as proposed by INNIS and GELFAND (1990), 96^{o}C for 15 sec in contrary to the

normally used denaturation of 94^{o}C for 1min. Generally PCR works with denaturing temperatures of 91-97^{o}C (RYBICKI, 2001).

1.4 Annealing temperature and primer design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification. Both the length and increasing (G+C) content increase the Tm (see formula below) of the NA duplex. This implies that the annealing temperature (Ta) chosen for a PCR depends on length and composition of the primer.

$$Tm = 4(G+C) + 2(A+T)\ ^{o}C$$

INNIS and GELFAND (1990) recommended the use of Ta about 5^{o}C below the lowest Tm of the pair of primers to be used. RYCHLIK *et al.* (1990) explained that if the Ta is increased by 1^{o}C every other cycle, specificity of amplification and yield of products <1 kb in length are both increased. If the Ta is too low there is a problem of primers annealing to sequences other that the true target or mismatching, which result in reduction of yield of desired products (RYBICKI, 2001). Too high Ta also results in too little product.

1.5 Elongation temperature and time

In most commonly used PCR procedures this is between 70-72^{o}C for 0.5-3 min, but 72^{o}C for 5 min in this study gave better amplification. Practically elongation occurs from the moment of annealing, but at about 70^{o}C activity becomes optimal and primer extension occurs up to 100 bases/sec (INNIS and GELFAND, 1990). Generally longer products require longer times and longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration, and when dNTP and/or primer depletion may become limiting (RYBICKI, 2001).

1.6 Cycle number

The starting concentration of target DNA largely affects the number of amplification cycles necessary to produce a band visible on a gel. INNIS and GELFAND (1990), for example, recommend from 40-45 cycles to amplify 50 target molecules, and 25-30 to amplify $3x10^5$ molecules to the same concentration. An explanation for this non-proportionality is the plateau effect when product reaches 0.3-1 nM. Factors, which possibly cause this include;

- Degradation of reactants (dNTP, enzyme);
- Reactant depletion (primers, with short products and dNTP's with long products);
- End product inhibition (pyrophosphate formation);
- Competition for reactants by non specific products; and
- Competition for primer binding by re-annealing of concentrated products (INNIS and GELFAND, 1990).

Plateau effect is the attenuation in the exponential rate of product accumulation in late stages of a PCR (RYBICKI, 2001).

2. Interpreting and analyzing data from molecular screening techniques

SOLTIS *et al*., (1992) stressed the importance of understanding the different ways that data from molecular techniques can be utilized. Before embarking upon a programme of applying any one of the techniques to a diversity and/or variability study, critical selection of an appropriate statistical package should be made for data collection and analysis. Molecular data are collected in two fundamentally different ways, namely, as discrete characters or as continues characters.

The data derived from PCR have their strength in distinguishing individuals, cultivars or accessions. The difficulty of achieving a robust profile in these types of data, particularly in RAPDs, makes the reliability for typing questionable. The presence or absence of bands, however, can be scored and data is converted to similarity matrices for calculation of genetic distance between populations (ELLSWORTH *et al*., 1993) or the presence or absence of bands could be scored and compared to a standard Molecular Weight Marker as employed in this study.