REGULATION OF HYPERHYDRICITY IN *ALOE POLYPHYLLA* PROPAGATED *IN VITRO*

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Research Centre for Plant Growth and Development
School of Biological and Conservation Sciences
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Student Declaration

Regulation of hyperhydricity in *Aloe polyphylla* propagated *in vitro*

I, MARIYANA VASILEVA IVANOVA, Student Number 200271768

declare that:

(i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg;

(ii) This dissertation has not been submitted for any degrees or examination at any other University;

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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Chapter 5 **HYPERHYDRICITY IN SHOOT CULTURES OF *ALOE POLYPHYLLA* CAN BE EFFECTIVELY REDUCED BY VENTILATION OF CULTURE VESSELS**

Abstract

Micropropagation of *Aloe polyphylla*, an endangered species with a high ornamental and medicinal value, is an important part of its conservation. However, the *in vitro* culture was hindered by the phenomenon of hyperhydricity. The research reported in this thesis was undertaken for two reasons. Firstly, to understand the role of various culture factors involved in the process of hyperhydricity in *A. polyphylla* and to identify the *in vitro* conditions, under which this disorder can be prevented. Secondly, we conducted an investigation into the underlying mechanisms of this phenomenon by probing if it was mediated through internal cytokinins.

Ammonium (NH_4^+) ions, applied cytokinins (CKs) and CK concentrations were tested in multifactorial combinations and significantly influenced the regeneration rate and occurrence of hyperhydricity. Shoots were grown on media with different NH_4^+ concentrations (10.3, 20.6 and 61.8 mM) and supplemented with BA, zeatin or TDZ at 0, 5 or 15 µM. Elevating the levels of NH_4^+, in the absence of CKs, could not induce hyperhydricity. Similarly, very low hyperhydricity was observed when CKs were added to media containing low NH_4^+ (10.3 mM). However, in the presence of higher NH_4^+ concentrations, CKs increased hyperhydricity in a concentration-dependant manner, suggesting that they were capable of inducing this syndrome only when other factors in the culture system were not optimised. High numbers of healthy looking shoots were produced on media with low NH_4^+ and low BA or zeatin (5 µM). The use of TDZ resulted in the formation of buds, which did not develop into shoots.

In view of the fact that NH_4^+ was supplied in the form of NH_4NO_3, it was difficult to determine if NH_4^+ or nitrate (NO_3^-) ions were associated with the increase in hyperhydricity. We further examined the role of nitrogen (N) supplied as inorganic NH_4^+ or NO_3^-, or organic glutamine. The omission of total N from the culture medium resulted in low multiplication and hindered shoot growth. Ammonium as the sole source of N depressed shoot regeneration and growth and escalated the frequency of hyperhydricity to *ca.* 50%. When NO_3^- was used as the sole N source, shoots of fine quality were produced and hyperhydricity was completely eliminated. Overall, the

MS N mix was superior to any single N source for multiplication and growth of shoots, suggesting a synergistic effect between NH_4^+ and NO_3^- on shoot regeneration. Furthermore, not only the absolute amount of N, but also the relative amounts of NH_4^+ and NO_3^- influenced the multiplication rate, frequency of hyperhydricity and shoot quality. The highest regeneration was obtained with NH_4^+ : NO_3^- ratios (mM) of 20 : 40, 30 : 30 and 40 : 20. Decreasing the ratio of NH_4^+ : NO_3^- lowered the occurrence of hyperhydricity. The potential of glutamine as the sole source of N was also demonstrated, since its application resulted in the production of good quality shoots and almost no hyperhydricity.

Shoot explants grown in static liquid media became hyperhydric and lost their ability to regenerate. The type of gelling agent used to solidify the medium affected greatly hyperhydricity and shoot multiplication. Gelrite resulted in a significantly lower multiplication rate and four times higher hyperhydricity (64.7%) compared to when agar was used. Gelrite was further selected to test the hypothesis if hyperhydricity can be overcome by decreasing the relative matric potential of the media, and respectively the availability of water, as represented by increasing gelrite concentrations. Satisfactory reduction in hyperhydricity was achieved only at 16 g l^{-1} gelrite, however the regeneration also decreased. The nature of the gelling agent is therefore essential for the successful control of this phenomenon.

It appears that a crucial prerequisite for the reduction of hyperhydricity in tissue cultures of *A. polyphylla* is the gaseous exchange between the *in vitro* atmosphere and the outside environment. In ventilated cultures, achieved by using a modified lid with a hole (d = 7 mm) covered with polyester or cotton mesh, hyperhydricity was completely eliminated, irrespectively of the type of gelling agent. Ventilation was further advantageous for the *in vitro* regenerants by increasing their leaf chlorophyll content as well as epicuticular wax deposition, the last one being indicative of the development of the water loss regulation mechanisms of explants. The increased culture ventilation, however, was negatively correlated with the regeneration rate and shoot growth.

Endogenous CKs were measured in *in vitro* regenerants after an eight-week cycle to examine whether the hyperhydricity-inducing effect of exogenous CKs and gelling

agents is associated with changes in the endogenous CK content. The content of endogenous CKs, determined by HPLC-mass spectrometry, in the shoots grown on CK-free media comprised isopentenyladenine-, *trans*-zeatin- and *cis*-zeatin-type CKs. The application of exogenous CKs resulted in an increase in the CK content of the shoots. Following application of zeatin, dihydrozeatin-type CKs were also detected in the newly-formed shoots. Application of BA to the media led to a transition from isoprenoid CKs to aromatic CKs in the shoots. Shoots grown on gelrite media contained higher levels of endogenous CKs compared to those on agar media. Total CK content of hyperhydric shoots was higher than that of normal shoots grown on the same medium. We suggest that the ability of exogenous CKs and gelrite to induce hyperhydricity in shoots of *Aloe polyphylla* is at least partially due to up-regulation of endogenous CK levels. However, hyperhydricity is a multifactor process in which different factors intervene.

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ψ_g	gravitational potential
ψ_m	matric potential
ψ_p	pressure potential
ψ_s	osmotic potential
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ANOVA	analysis of variance
BA	N^6-benzyladenine
BA9G	N^6-benzyladenine 9-glucoside
BAR	N^6-benzyladenine 9-riboside
BARMP	N^6-benzyladenine 9-riboside-5'-monophosphate
CK	cytokinin
*c*Z	*cis*-zeatin
*c*ZOG	*cis*-zeatin O-glucoside
*c*ZR	*cis*-zeatin 9-riboside
*c*ZRMP	*cis*-zeatin 9-riboside-5'-monophosphate
*c*ZROG	*cis*-zeatin 9-riboside O-glucoside
DHZ	dihydrozeatin
DHZ9G	dihydrozeatin 9-glucoside
DHZOG	dihydrozeatin O-glucoside
DHZR	dihydrozeatin 9-riboside
DHZRMP	dihydrozeatin 9-riboside-5'-monophosphate
DHZROG	dihydrozeatin 9-riboside O-glucoside
DMAPP	dimethylallyl diphosphate
DMF	*N,N*-dimethylformamide
DW	dry weight
ESEM	environmental scanning electron microscope
FW	fresh weight
HPLC	high performance liquid chromatography

HS	hyperhydric shoots
IAA	indole-3-acetic acid
IAC	increase of agar concentration
IACh	immunoaffinity chromatography
IBA	indole-3-butyric acid
IGC	increase of gelrite concentration
iP	N^6-(Δ^2-isopentenyl)adenine
iP9G	N^6-(Δ^2-isopentenyl)adenine 9-glucoside
iPR	N^6-(Δ^2-isopentenyl)adenine 9-riboside
iPRMP	N^6-(Δ^2-isopentenyl)adenine 9-riboside-5'-monophosphate
LC(+)ES-MS	liquid chromatography + electrospray ionization–mass spectrometry
LSD	least significant difference
MES	2[*N*-morpholino]ethanesulphonic acid
MS	Murashige and Skoog (1962) medium
*m*T	*meta*-topolin
*m*T9G	*meta*-topolin 9-glucoside
*m*TOG	*meta*-topolin O-glucoside
*m*TR	*meta*-topolin 9-riboside
*m*TRMP	*meta*-topolin 9-riboside-5'-monophosphate
*m*TROG	*meta*-topolin 9-riboside O-glucoside
NAA	α-naphthaleneacetic acid
nd	not detected
NS	normal shoots
*o*T	*ortho*-topolin
*o*T9G	*ortho*-topolin 9-glucoside
*o*TOG	*ortho*-topolin O-glucoside
*o*TR	*ortho*-topolin 9-riboside
*o*TRMP	*ortho*-topolin 9-riboside-5'-monophosphate
*o*TROG	*ortho*-topolin 9-riboside O-glucoside
PAL	phenylalanine ammonia-lyase
PGRs	plant growth regulators

PPFD	photosynthetic photon flux density
*p*T	*para*-topolin
*p*TOG	*para*-topolin O-glucoside
*p*TR	*para*-topolin 9-riboside
*p*TRMP	*para*-topolin 9-riboside-5'-monophosphate
*p*TROG	*para*-topolin 9-riboside O-glucoside
ROS	reactive oxygen species
RWL	relative water loss
SE	standard error of mean
T	topolin
TDZ or Thidiazuron	N-phenyl-N´-1,2,3-thiadiazol-5-yl urea
*t*Z	*trans*-zeatin
*t*ZOG	*trans*-zeatin O-glucoside
*t*ZR	*trans*-zeatin 9-riboside
*t*ZRMP	*trans*-zeatin 9-riboside-5'-monophosphate
*t*ZROG	*trans*-zeatin 9-riboside O-glucoside
Z	zeatin
Z9G	zeatin 9-glucoside

Chapter 1

INTRODUCTION

1.1 The genus *Aloe*

The tribe *Aloineae* is fundamentally a South African group, but some of the genera included in it are also found elsewhere. The tribe is rather small and variable, since not all authors include the same genera in it (RILEY and MAJUMDAR, 1979). According to RILEY and MAJUMDAR (1979) the tribe consists of the following genera: *Aloe*, *Gasteria*, *Haworthia*, *Astroloba*, *Poellnitzia*, *Chamaealoe*, *Chortolirion*, *Lamatophyllu*, *Leptaloe* and *Guillauminia*.

The name Aloe appears to be derived from an old Greek name for the plant, similar to the Hebrew word 'Allal' meaning bitter, although it may also have its origins in the Arabic word 'Alloch'. Medicinal aloes were known to the Greeks from the island of Socotra as early as 4 BC, while the plant was cultivated in pots in the days of Rome and Naples (POOLEY, 1998). The genus *Aloe* occurs mainly in Africa, but is introduced into many other parts of the world. A number of ornamental species of this genus are widely cultivated in Europe and the New World for their foliage and decorative effect in gardens, parks and public buildings. Aloes form a conspicuous part of the South African landscape and their striking beauty is appreciated by many people.

1.1.1 Distribution and morphology

The genus is found in southern and tropical Africa, Madagascar, Arabia, the Mediterranean region, Socotra, India and China (POOLEY, 1998). According to REYNOLDS (1966), there are 324 known species of *Aloe*, of which 136 occurred in South Africa, 142 over the rest of Africa, Socotra and Arabia, and 46 in Madagascar. HARDING (1980) listed 363 known species and by 1989 there where 375 species (WEST, 1992). This distribution sheds some light on their origin. As *Aloe* is not

represented in South America, Australia and Antarctica, but occurs in Madagascar, it seems that the genus originated in Africa after the break up of Gondwanaland but before Madagascar was separated from Africa (WEST, 1992).

Aloe is a large genus. The plants may be herbaceous and about 23 cm tall (as in *Aloe myriacantha*) or arborescent and about 15 metres tall (as in *Aloe eminens*) and may be stemless or caulescent. If stems are present they are simple or branched. The leaves are fleshy, succulent and many-ranked. They may be toothed or prickly on the margins. They are usually crowded in a dense rosette, which is acaulescent and on the ground or may occur at the end of a short or long stem. The flowers are in a raceme or an unbellate or subcorymbose panicle and occur at the end of a peduncle, which may be of considerable length. The fruit is a coriaceous trigonous capsule. Seeds are flattened or three-angled, often winged. The flowers are generally numerous, usually bright red or yellow, and beautiful. The rootstock is a rhizome and is not bulbous (RILEY and MAJUMDAR, 1979).

1.1.2 Medicinal uses

The juice of Aloes has been used medicinally for centuries. It is said that Alexander the Great conquered the island of Socotra to gain control over the main supply of aloetic medicine. To this day, most of the aloe drugs in the world are produced from the famous *Aloe vera* (*Aloe barbadensis*). This species originally came from North Africa or Arabia but is widely cultivated in many parts of the world. The leaves of *Aloe vera* are the source of bitter sap. Commercially, the product is known as Curacao Aloes or Barbados Aloes (VAN WYK and SMITH, 1996). After collection, the leaf sap is evaporated and cooled to form a compacted yellowish, reddish or black powder. The active principle of the powder is a glycoside used in medicine mainly as a purgative and vermifuge, and in small quantities in bitters (HEYWOOD and CHANT, 1982).

In South Africa, *Aloe ferox* is the source of a similar medicinal product known as Aloe lump or Cape Aloes. This purgative drug is used for stomach complaints, mainly as a laxative to ‘purify’ the stomach. It is a dark brown resinous solid and has been commercially utilised for centuries. When an aloe leaf is cut, extremely bitter yellow

juice oozes from small canals situated just below the surface in the green part of the leaf. The fresh juice is widely used as a first-aid treatment for burn wounds, while the inner leaf pulp is processed into aloe gel. To produce the medicinal product called Cape Aloes, the yellow juice is collected and dried by an age-old method. The main purgative principle is the anthrone *C*-glucoside aloin (barbaloin; VAN WYK and SMITH, 1996).

Aloe ferox and other species are used to a great extent in traditional human and livestock medicines. The leaves or roots, boiled in water, are taken as a laxative, but also for arthritis, eczema, conjunctivitis (WATT and BREYER-BRANDWIJK, 1962), hypertension and stress (PUJOL, 1990). Leaf sap of several species such as *Aloe arborescens* and *Aloe greatheadii* is applied externally to treat skin irritations, bruises and burns. The dry leaves of *Aloe marlothii* are popular in snuff mixtures (VAN WYK *et al.*, 1997).

1.1.3 Cosmetic uses

In recent years, there has been a growing interest in aloe products, particularly aloe gel for the cosmetic industry. The source of most of the products is the well known exotic species *Aloe vera*. To produce the product known as aloe gel or aloe pulp, the fleshy inner portion of the leaf (the so called white juice) is carefully separated from the outer green part, which contains the bitter yellow exudate. The fleshy part is tasteless, and is simply crushed and homogenised to form the gel. Aloe gel is a watery mixture of pectic carbohydrates, amino acids, minerals, trace elements, organic acids and various minor compounds such as enzymes, claimed to be ‘biologically active’. The gel is used in the health food industry to produce tonics, but the main commercial application is in the lucrative cosmetic industry. It is now also possible to buy cosmetics made from the leaf gel of the indigenous *Aloe ferox* (VAN WYK and SMITH, 1996).

1.1.4 Conservation status

The interest in aloes, which developed after the publication of “Aloes of South Africa” by Dr Reynolds, was not confined to aloes in the veld. People collected plants to

grow in their gardens, but no great harm was done until the attention of commercial collectors was attracted. Then the number of plants taken from the veld increased very steeply. Aloes acquired a monetary value and unfortunately, because higher prices can be obtained for them, rare species, species with restricted distribution and plants from type localities are in particular demand. A number of species are threatened and if nothing is done to curb their exploitation they will become extinct in the wild even if they survive in gardens (WEST, 1992).

With few exceptions, aloes are protected by environmental legislation in all the provinces of South Africa. Although some species have never had large populations, various factors have contributed to the decline in numbers of several species. These factors include urban and industrial expansion, agricultural development, aforestation and mining activities. Nurseries specialising in indigenous and/or succulent plants should be sourced for propagation material (VAN WYK and SMITH, 1996).

1.2 *Aloe polyphylla*

Aloe polyphylla was first discovered on Phurumela Mountain in November 1915 by Mr FH Holland who sent plants and material to Dr Schönland at the Albany Museum, Grahamstown. Schönland drew up a description of his *A. polyphylla* in January 1923 but never published it. It was not until 1934 that Mr NS Pillans described the species from Schönland's notes and from photographs and material (REYNOLDS, 1950).

Few have seen *A. polyphylla* in its natural habitat, and it appears to be the least known, one of the rarest, and one of the most unique of all the aloes. The most striking feature is the arrangement of the leaves, which are always in five ranks of 15-30 leaves each, and spirally twisted in a clockwise or anti-clockwise manner. The leaves are about 150, 20-30 cm long and 6-10 cm wide. They are ovate-oblong, acuminate, ascending incurved and with a grey-green colour. The marginal teeth are soft and white, and are not arranged in any regular manner. In some leaves, marginal teeth occur in the lower half only, and in others in the upper half. Sometimes there are more teeth on one margin than the other, while the interspaces vary considerably. Another interesting character is the white raised keel along the lower leaf surface, which is never in the middle but always nearer the one or other margin.

The keel is most noticeable in young plants, becoming less defined in older plants, and almost inconspicuous in the largest specimens (REYNOLDS, 1950). Another distinctive characteristic is the leaf tip, which becomes dry and turns purplish-black. The inflorescence is up to 60 cm high and is branched low down on the plant into 3 to 8 racemes. Each raceme is densely flowered, with tubular pale red to salmon-coloured flowers of up to 6 cm long. Flowering occurs mainly in September to November (VAN WYK and SMITH, 1996).

The type locality of *A. polyphylla* is the Western slopes of Phurumela Mountain, Lesotho and the Drakensburg range in South Africa. The aloes grow at an altitude of about 2400 metres, and are often under snow in winter. They grow on steep slopes, sometimes solitary but usually in dense groups of a dozen or more individuals. They do not increase from suckers or off-shoots, but occasionally plants divide into two and three rosettes. The plants are stemless. Large specimens may have very short stems (REYNOLDS, 1950).

Aloe polyphylla does not take kindly to cultivation away from its natural habitat, and transplants whether young or old almost invariably die.

Aloe polyphylla is an endangered species. The removal and export from Lesotho of both plants and seeds is prohibited by Resident Commissioner's Notice of the 20th September 1938 (REYNOLDS, 1950).

'Polyphylla' literally means 'many leaves'. The Afrikaans common name is Kroonaalwyn (VAN WYK and SMITH, 1996).

1.3 Tissue culture of *Aloe* species

A limited amount of work has been done on tissue culture of *Aloe* species. GROENEWALD *et al.* (1975) reported the successful establishment of calii and subsequent plant regeneration in *Aloe pretoriensis* Pole Evans from seed tissues. RACCHI (1987) cultured different explants of *Aloe ferox* Mill. and produced callus from seed tissues which, when subcultured on the same medium, produced clusters of somatic embryos which developed into plantlets. Very low morphogenetic ability

was reported for *Aloe barbadensis* (SANCHEZ *et al.*, 1988), correlated with variations in nuclear DNA content occurring throughout the culture: only strictly diploid calli showed morphogenetic ability. However, NATALI *et al.* (1990) established a rapid and highly effective method for micropropagation of *Aloe barbadensis* Mill. from vegetative meristems. Apices were cultured on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin for 15 – 30 days. High morphogenetic ability was maintained by transferring the explants to media with 2,4-D and N^6-benzyladenine (BA). The effects of different plant growth regulators (PGR), temperature and sucrose concentration on the formation of multiple buds in *Aloe barbadensis* were investigated by MEYER and VAN STADEN (1991). Maximal bud growth and rooting of shoots was obtained on a medium supplemented with 5 µM indole-3-butyric acid (IBA). Medium with IBA was superior to medium with α-naphthaleneacetic acid (NAA), while the sole addition of indole-3-acetic acid (IAA) resulted in the formation of axillary but not adventitious buds. Plant morphogenesis was inhibited by 2,4-D, whereas kinetin, BA and TDZ were toxic to the explants. The optimal temperature for bud growth and development was 25 ℃ and the optimal sucrose concentration was 30 g l^{-1}. ROY and SARKAR (1991) obtained shoots from calii of *Aloe vera*. Callus formation was induced in stem segments from young shoots on medium containing 2,4-D and kinetin. Shoots were initiated from the calii at reduced 2,4-D and increased kinetin concentrations. RICHWINE *et al.* (1995) reported that shoot cultures of *Aloe*, *Gasteria* and *Haworthia* species were initiated directly from immature inflorescences. Explants placed on a medium containing 5.4 µM zeatin riboside formed shoots within 8 to 12 weeks and long-term shoot cultures were maintained either on the same medium or medium supplemented with 4 µM BA.

1.3.1 Tissue culture of Aloe polyphylla

The *in vitro* culture of *A. polyphylla* was established from seeds by ABRIE and VAN STADEN (2001). In this preliminary report only BA was used for shoot regeneration and the problem of hyperhydricity was reported as a major hindrance to the micropropagation. In a subsequent study aiming at optimisation of the tissue culture protocol, the effects of various cytokinins as well as temperature and sucrose concentration on shoot multiplication were evaluated (CHUKWUJEKWU *et al.*, 2002). The application of zeatin, alone or in combination with IBA or NAA, resulted in very

low hyperhydricity. BAIRU *et al.* (2007) suggested the use of *meta*-topolin as a replacement of conventionally used BA and zeatin to control the process of hyperhydricity *in vitro*.

1.4 Hyperhydricity

1.4.1 Introduction

When the 'glassy shoot' syndrome was first recognised it was described as vitrification, glassiness, translucency, succulency and hyperhydration (ZIV, 1991). The term vitrification, although used very widely in the literature, was said to be misused since it refers to a physical and not a biological process (ZIV, 1991). Instead the term hyperhydricity was proposed (DEBERGH *et al.*, 1992). Hyperhydricity has been reported to affect both woody (BORNMAN and VOGELMANN, 1984; KATAEVA *et al.*, 1991; KADOTA and NIIMI, 2003) and herbaceous plants *in vitro* (LESHEM *et al.*, 1988; ZIV and ARIEL, 1992; PICOLI *et al.*, 2001).

Manifested mainly in the leaves, hyperhydricity affects the two major processes carried out by the leaves, namely photosynthesis and gas exchange (DEBERGH and MAENE, 1984). Hyperhydric leaves appear water-soaked, glassy and translucent. Shoots, affected by this disorder often form callus at their bases and no rooting occur (Figure 1.1). The establishment of hyperhydric shoots to the *ex vitro* environment is hampered by the lack of roots and poorly developed leaf mechanisms for control of water loss. Apart from the visual morphological symptoms, by which hyperhydricity is traditionally defined, this phenomenon causes changes in the plant system at anatomical and physiological levels.

1.4.2 Anatomical and physiological characteristics of hyperhydric plants

Microscopic investigations of hyperhydric leaves revealed a complete absence or a reduced number of palisade cell layers (BRAINERD and FUCHIGAMI, 1981; OLMOS and HELLÍN, 1998) and an unorganized spongy mesophyll with large intercellular spaces (WERKER and LESHEM, 1987; PICOLI *et al.*, 2001). NMR imaging suggested that the excess water associated with hyperhydric plants was located in

the intercellular spaces (GRIBBLE *et al.*, 1997). Hyperhydric cells had thin walls and their cytoplasm was largely vacuolated (LESHEM, 1983a). In *Eucalyptus saligna*, the number of chloroplasts in hyperhydric cells was lower and they showed reduced thylakoid stacking (JONES *et al.*, 1993), while in sweetgum the chloroplasts lacked normal organization into grana and stroma (WETZSTEIN and SOMMER, 1982).

Figure 1.1: Normal shoots (NS; left) and hyperhydric shoots (HS; right) of *Aloe polyphylla*. Leaves of HS are thick, water-logged and translucent. HS form callus on their bases and no rooting is observed. Bar = 10 mm.

The surface of hyperhydric leaves is often characterized by a thin epidermis with perforations, scattered or no deposition of epicuticular wax and abnormally large stomata, with damaged guard cells (ZIV, 1991; SATO *et al.*, 1993; OLMOS and HELLÍN, 1998; PICOLI *et al.*, 2001). The deformations in the guard cell walls could have resulted from loss of elasticity or from abnormal orientation of cellulose microfibrils (ZIV and ARIEL, 1994). Stomata of hyperhydric leaves did not close in response to different signals, such as darkness, abscisic acid (ABA), high CO_2 or Ca^{2+} (ZIV *et al.*, 1987).

Hyperhydric stems were found to exhibit reduced lignification, hypertrophy of parenchyma tissue, large apoplastic spaces and abnormal organization of vascular bundles (LESHEM, 1983a; VIEITEZ *et al.*, 1985; LETOUZÉ and DAGUIN, 1987).

The altered morphology and anatomy of hyperhydric plants were found to influence various physiological parameters and processes. A significant increase in water content, as indicated by decreased dry weight, was observed in hyperhydric tissues (KEVERS *et al.*, 1984; SAHER *et al.*, 2004). Hyperhydricity has been associated with lower chlorophyll content (PHAN and LETOUZÉ, 1983; ZIV *et al.*, 1983; JONES *et al.*, 1993; SAHER *et al.*, 2004), reduced photosynthetic rate (JONES *et al.*, 1993; FRANCK *et al.*, 2001), decreased protein content as well as activities of enzymes involved in the lignin synthesis (PHAN and LETOUZÉ, 1983; KEVERS *et al.*, 1984; ZIV, 1991), less lignin and cellulose (KEVERS *et al.*, 1987; SAHER *et al.*, 2004) and high levels of ethylene evolution (KEVERS and GASPAR, 1985b; ZIV and ARIEL, 1992). Recent evidence suggests that oxidative stress, involving reactive oxygen species (ROS), is involved in the induction of hyperhydricity (FRANCK *et al.*, 1995; OLMOS *et al.*, 1997; PIQUERAS *et al.*, 1998; CHEN and ZIV, 2001). SAHER *et al.* (2004) reported the generation of toxic levels of H_2O_2 in hyperhydric carnation tissues, implying that they could be responsible for some of the abnormalities observed in the hyperhydric shoots, namely high lipid peroxidation and membrane damage.

Increase in the concentration of endogenous cytokinins in hyperhydric plants has been consistently reported in the literature (KATAEVA *et al.*, 1991; DANTAS de OLIVEIRA *et al.*, 1997; VANDEMOORTELE *et al.*, 2001). However, it is possible to control hyperhydricity in shoots by modulating the internal cytokinin types through the nature of the exogenous cytokinin applied (VANKOVA *et al.*, 1991) or by an osmotic pre-treatment (VANDEMOORTELE *et al.*, 2001). Hyperhydricity of *in vitro* regenerants of grass pea was even correlated with an abnormal DNA content (OCHATT *et al.*, 2002). Nevertheless, this finding was not consistent with the results obtained from DNA analysis of hyperhydric shoots of *Picea abies* and *Prunus avium*, which showed no abnormalities (HAKMAN *et al.*, 1984; FRANCK *et al.*, 2004). Also, hyperhydric conditions have been shown to induce changes in protein synthesis, thus affecting interrelated metabolic pathways (ZIV, 1991). An approximately 30 – 31 kDa

protein was present in hyperhydric but not in normal leaves of carnation (ZIV, 1991; ZIV and ARIEL, 1992). The synthesis of stress-induced binding protein (BiP) was significantly increased in hyperhydric shoots of pepper and eggplant (FONTES *et al.*, 1999; PICOLI *et al.*, 2001).

The anatomical and physiological characteristics of hyperhydric plants are similar for many species. This suggests that while many factors of the *in vitro* environment may cause hyperhydricity (see below), the consequent perturbation of the morphogenetic processes stems from a common event. KEVERS *et al.* (1984) proposed a hypothesis according to which the increased ethylene production was this 'key' event. The various non-wounding stress conditions would mediate a rapid endogenous ethylene burst and the gas would retroinhibit its own synthesis subsequently by repressing the formation of ACC-synthase. The reduced ethylene production would then decrease the activities of phenylalanine ammonia-lyase (PAL) and acidic peroxidases. Since PAL and acidic peroxidases are involved in the process of cell wall lignification, their low activities would explain hypolignification found in hyperhydric plants (KEVERS *et al.*, 1984 and references therein). A parallel decrease in cellulose synthesis would also be expected. Excess of NH_4^+ in the medium would decrease the C/N ratio and bring about a diversion of carbohydrates to amino acids. A higher activity of glutamate dihydrogenase in hyperhydric plants (DAGUIN and LETOUZÉ, 1986) indicates a higher incorporation of NH_4^+ into organic compounds. Deficiency of both lignin and cellulose would allow more water uptake due to reduced cell wall pressure and cause the hyperhydric abnormalities (KEVERS *et al.*, 1984; GASPAR *et al.*, 1987).

1.4.3 Factors involved in hyperhydricity

Hyperhydricity is a multifactor process and a number of factors have been ascribed as being responsible for it. However, many of these factors will induce hyperhydricity when other conditions in the culture system are not optimized (DEBERGH *et al.*, 1992). A brief description of the various factors involved in the process of hyperhydricity is given below and more detailed explanations are presented in the Discussion section of the respective Chapters.

1.4.3.1 Culture medium

The physical and chemical state of the medium influence greatly plant development. Liquid media consistently gave rise to hyperhydricity (KEVERS and GASPAR, 1985b; HDIDER and DESJARDINS, 1993; MAJADA *et al.*, 1997; WHITEHOUSE *et al.*, 2002). The semi-solid state is achieved by the addition of gelling agents, the most common of which are agar and gelrite. The gelling agent affects the physical parameters of the medium by influencing the matric potential of the medium and thus, the availability of water and other substances to the explants. On the other hand, the chemical state of the media is also affected by the impurities introduced together with the gelling agent. The type and concentration of the gelling agent are important factors in the control of hyperhydricity. Increasing the concentration of gelling agent reduced hyperhydricity but very often also decreased shoot regeneration (VON ARNOLD and ERIKSSON, 1984; WILLIAMS and TAJI, 1991; CASANOVA *et al.*, 2008). High gelling agent concentrations reduced the availability of various medium components, in particular cytokinins (DEBERGH, 1983; BORNMAN and VOGELMANN, 1984). The type of gelling agent has a significant effect on the performance of plants *in vitro*. Gelrite was found to increase hyperhydricity in few species (PASQUALETTO *et al.*, 1986; TSAY, 1998; FRANCK *et al.*, 2004). In apple, gelrite always produced hyperhydric leaves even though media gelled with 1.5 g l^{-1} gelrite had the same gel strength as using 7.0 g l^{-1} agar, which did not induce hyperhydricity (PASQUALETTO *et al.*, 1988a). However, this may result from the presence of substances in the agar, which are responsible for hydric control (NAIRN *et al.*, 1995).

Shoot regeneration *in vitro* and plant micropropagation require a continuous supply of growth regulators to the culture medium. Few studies have identified cytokinins to be the primary inducer of hyperhydricity (LESHEM *et al.*, 1988; FRICK, 1991). However, in globe artichoke BA was found to induce this phenomenon when other culture conditions were adverse, i.e. high relative humidity and high matric potential (DEBERGH, 1983). Cytokinins were also shown to increase hyperhydricity in a concentration dependant manner (DEBERGH, 1983; KATAEVA *et al.*, 1991; WILLIAMS and TAJI, 1991). Hyperhydricity in melon could be reversed by reducing the cytokinin level (LESHEM *et al.*, 1988). The type of cytokinin is also essential.

Substituting BA and zeatin with *meta*-topolin resulted in elimination of hyperhydric shoots in tissue cultured *Aloe polyphylla* (BAIRU *et al.*, 2007). The effects of auxins on hyperhydricity are less studied and very often related to cytokinins. LESHEM (1983b) suggested that the imbalance of auxins and cytokinins induced hyperhydricity. GASPAR *et al.* (1987) correlated the effects of auxins, IAA in particular, to ethylene.

The mineral composition of the media was also shown to influence hyperhydricity in some species. At concentration of 20.6 mM, as in MS medium (MURASHIGE and SKOOG, 1962), NH_4^+ ions promoted hyperhydricity (DAGUIN and LETOUZÉ, 1986; ZIV *et al.*, 1987; BRAND, 1993). Excess NH_4^+ may modify the activities of enzyme involved in the synthesis of lignin and may divert carbohydrates away from the lignin and cellulose biosynthesis. Reducing the level of NH_4^+ in the medium correlated with increased lignification and decreased hyperhydricity in chestnut and willow (VIEITEZ *et al.*, 1985; LETOUZÉ and DAGUIN, 1987). In plum, Cl^- ions were shown to be involved in hyperhydricity (QUOIRIN and LEPOIVRE, 1977). In apple, lowering the concentration of K^+ in medium with gelrite produced more hyperhydric shoots, affected tissue appearance and altered shoot metabolic activity (PASQUALETTO *et al.*, 1988a).

There are some contradictory results in the literature regarding the effect of carbohydrates on hyperhydricity, which appears to be species-dependant. When the concentration of sucrose in the medium was higher than 30 g l^{-1}, carnation shoots produced fewer 'glassy' leaves (ZIV *et al.*, 1983). Increased sucrose, however, did not reduce the number of hyperhydric shoots in globe artichoke (DEBERGH *et al.*, 1981) and even promoted hyperhydricity in *Petunia* leaves (ZIMMERMAN and COBB, 1989). RUGINI *et al.* (1987) tested various carbohydrates in almond and olive and found that 45 g l^{-1} fructose decreased hyperhydricity significantly.

1.4.3.2 Culture atmosphere

High relative humidity and high concentrations of ethylene and CO_2 in the gaseous phase of the culture vessel were found to play a major role in the occurrence of hyperhydricity. Reducing the relative humidity in the culture atmosphere, by using

various methods (see Chapter 5), reduced the production of hyperhydric shoots (SHORT *et al.*, 1987; TANAKA *et al.*, 1992a; PÉREZ-TORNERO *et al.*, 2001; MAKUNGA *et al.*, 2006). A simple technique to overcome hyperhydricity is the increased aeration of the culture vessel, which can be achieved for exapmple by using permeable membranes (DILLEN and BUYSENS, 1989). Ventilating the vessels reduces the relative humidity and increases the gaseous exchange between the culture headspace and the outer atmosphere, thus avoiding the accumulation of toxic levels of ethylene and CO_2. Increased ventilation reduced hperhydricity in a number of species (HAKKAART and VERSLUIJS, 1983; MAJADA *et al.*, 1997; LAI *et al.*, 2005; CASANOVA *et al.*, 2008). However, a negative correlation between ventilation and shoot multiplication and growth was also observed. Apart from overcoming hyperhydricity, ventilation of culture vessels also achieves induction of autotrophy in tissue cultures and *in vitro* hardening or acclimatization (KOZAI and SMITH, 1995; ZIV, 1995). The gas concentrations and composition in the atmosphere of the culture container are influenced not only by the ventilation but also by the volume of the medium in relation to the volume of the headspace and by the number of explants (DEBERGH *et al.*, 1992).

1.4.3.3 Environment

A number of factors of the growth room environment can influence plant growth and development *in vitro*, including the occurrence of hyperhydricity: temperature, light intensity, relative humidity of the growth room and air movement. Hyperhydricity tends to be promoted by high temperature, low light irradiance, or by placing cultures in the dark (GEORGE, 1993). Shoots of *Aster* and *Chrysanthemum* became hyperhydric if grown under low irradiance (STIMART, 1986). In *Olearia microdisca*, placing the cultures at temperature of 5 ℃ reduced hyperhydricity, but also decreased the rate of shoot multiplication (WILLIAMS and TAJI, 1991). Low hyperhydricity could be achieved by incubating the cultures at low temperature only for a limited period of time (few days) and then returning them to room temperature (TAJI and WILLIAMS, 1989). The effect of temperature on hyperhydricity can also be indirect. Creating a temperature gradient within the culture container reduces the relative humidity *in vitro* (MAENE and DEBERGH, 1987; VANDERSCHAEGHE and DEBERGH, 1987). This can be achieved by placing the cultures on a cooled plate,

which causes the water vapour to condense on the agar medium and consequently reduces hyperhydricity.

1.4.3.4 Initial explant

The susceptibility to hyperhydricity varies with species, cultivar (MAJADA *et al.*, 1997; OCHATT *et al.*, 2002; SAHER *et al.*, 2004), length of time since the last subculture and the time since the culture was established (DEBERGH *et al.*, 1992).

1.5 OBJECTIVES OF THE STUDY

The research presented here had three main objectives:

1) To understand the role of various culture factors involved in the process of hyperhydricity in *in vitro* regenerants of *Aloe polyphylla*;

2) To identify the *in vitro* conditions, under which this disorder could be successfully reduced; and

3) To investigated if the induction of this multifactor process in *A. polyphylla* was mediated through modifications of the internal cytokinins.

Chapter 2

EFFECT OF AMMONIUM IONS AND CYTOKININS ON MULTIPLICATION RATE AND HYPERHYDRICITY OF *IN VITRO* REGENERATED SHOOTS OF *ALOE POLYPHYLLA*

2.1 Introduction

The performance and survival of the *in vitro* cultures of many plant species are often hampered by the phenomenon of hyperhydricity (DEBERGH *et al.*, 1992). The process of hyperhydricity is generally considered as reversible, and new normal shoots (NS) could be formed by hyperhydric shoots (HS) after their transfer to optimal culture conditions. However, maintaining the hyperhydrating conditions could lead to irreversible loss of regenerative ability of the tissue (GASPAR *et al.*, 2000) and other detrimental changes, and ultimately death. Hyperhydricity has therefore been regarded as a neoplastic step to generalised cancer (GASPAR *et al.*, 1991; ANONYMOUS, 1995). The poor survival rate of HS when transferred to *ex vitro* conditions has been attributed to their low rootability, and rapid water loss caused by malfunctioning stomata, and reduced or absent deposition of epicuticular wax (ZIV and ARIEL, 1994). These losses engendered by hyperhydricity limit the potential of *in vitro* techniques for mass propagation. The most frequently reported morphological, anatomical and biochemical markers of HS have been well documented in several reviews on the subject (GASPAR, 1991; ZIV, 1991; DEBERGH *et al.*, 1992). The translucent, glassy appearance of HS is generally due to an increased water content, located in the apoplastic spaces (GRIBBLE *et al.*, 1997), and chlorophyll deficiency (PHAN and LETOUZÉ, 1983; ZIV *et al.*, 1983; FRANCK *et al.*, 1997; SAHER *et al.*, 2004), which could lead to a lower photosynthetic capacity (FRANCK *et al.*, 2001). Recent investigations have linked the process of hyperhydricity with conditions of oxidative stress (OLMOS *et al.*, 1997; FRANCK *et al.*, 1998; CHEN and ZIV, 2001; SAHER *et al.*, 2004), increased levels of endogenous cytokinins (VANDEMOORTELE *et al.*, 2001; IVANOVA *et al.*, 2006), up regulation of stress-

related protein synthesis (FONTES *et al.*, 1999; PICOLI *et al.*, 2001), and at least in one species, with abnormal DNA content (OCHATT *et al.*, 2002). It is worth noting the contrasting paradigm, proposed by KEVERS *et al.* (2004), on the application of the stress-induced state-change concept to the phenomenon of hyperhydricity. According to their hypothesis, hyperhydricity and some of the changes associated with it are adaptations for survival of the explants in the *in vitro* culture conditions.

Aloe polyphylla (Schönland ex Pillans) has been listed as highly endangered in the Red Data List (HILTON-TAYLOR, 1996). This rare species, indigenous to Lesotho and the Drakensberg range in Natal, has a high ornamental value. Despite considerable legal protection, its numbers in the wild are rapidly declining due to the removal of plants and seeds by collectors, and low viability of its seeds owing to the fact that its natural pollinator, the Malachite sunbird, is endangered (EMANOIL, 1994). ABRIE and VAN STADEN (2001) established a rapid protocol for *in vitro* propagation of *A. polyphylla* for conservation purposes, and as well to provide a reliable source of plants and thereby alleviating pressure on wild populations. Applying the commonly used benzyladenine (BA) resulted in a high multiplication rate. However, the quality and survival of the shoots were impaired by the occurrence of hyperhydricity. This problem prompted the optimisation of the micropropagation protocol and CHUKWUJEKWU *et al.* (2002) suggested the use of zeatin, alone or in combination with auxin, for avoiding HS. However, the high cost of zeatin makes its use unpractical for mass propagation as it increases the price of the final product. A recent report from our laboratory (BAIRU *et al.*, 2007) showed the potential of *meta*-topolin as possible replacement for BA and zeatin in tissue cultures of *A. polyphylla*, and its capacity to control the problem of hyperhydricity. Apart from the use of this promising plant growth regulator, we successfully identified other conditions, reported here, under which hyperhydricity can be prevented, thus resulting in new inexpensive solutions for a high production of healthy plants.

Hyperhydricity is a multifactor process and several factors have been ascribed as being responsible for it (see GASPAR, 1991; ZIV, 1991; DEBERGH *et al.*, 1992 for a comprehensive list). However, as pointed out by DEBERGH *et al.* (1992), many of these factors would only act to induce hyperhydricity when other conditions in the culture system are not optimised. A number of investigations have reported on the

hyperhydricity-inducing tendency of exogenous cytokinins, usually in a concentration-dependent manner (DEBERGH, 1983; LESHEM *et al.*, 1988; KATAEVA *et al.*, 1991; WILLIAMS and TAJI, 1991; KATAOKA and INOUE, 1992; IVANOVA *et al.*, 2006). Modifying other conditions in the culture media and/or the confined environment have a counteracting effect and lead to a reduction in the occurrence of hyperhydricity; for example increasing the concentration of gelling agent (DEBERGH *et al.*, 1981; BORNMAN and VOGELMANN, 1984; BÖTTCHER *et al.*, 1988; WILLIAMS and TAJI, 1991), decreasing the concentration of NH_4NO_3 (DAGUIN and LETOUZÉ, 1985; ZIV *et al.*, 1987), improved vessel aeration (DILLEN and BUYSENS, 1989; MAJADA *et al.*, 1997).

Large quantities of ammonium ions, as present in the MS medium, increased hyperhydricity in different species (RIFFAUD and CORNU, 1981; LETOUZÉ and DAGUIN, 1983; VIEITEZ *et al.*, 1985; ZIV *et al.*, 1987; BRAND, 1993). The excess of NH_4^+ ions may alter the activities of some enzymes involved in the synthesis of lignin (LETOUZÉ and DAGUIN, 1987), and may induce a detoxification process (GIVAN, 1979), during which carbohydrates are diverted away from the cellulose and lignin biosynthetic pathways, and utilized in the production of amino acids (BEAUCHESNE, 1981).

The aim of the present investigation was to elucidate the relationship between ammonium ions, applied cytokinins, and their concentration in the induction of hyperhydricity in *in vitro* regenerated shoots of *Aloe polyphylla*. Conditions under which the development of HS can be prevented were successfully identified. The information obtained from the work reported here can not only be applied to improve mass propagation of other *Aloe* species with a high medicinal and/or ornamental value, but it also provides new insights into our understanding of the phenomenon of hyperhydricity in a succulent plant and hence increasing our capacity to control it.

2.2 Materials and Methods

2.2.1 Plant material

The *in vitro* culture of *Aloe polyphylla* was established from seedlings in our laboratory in 1999 (ABRIE and VAN STADEN, 2001). It was subsequently maintained and multiplied by subculturing the shoots at eight weekly intervals. They were grown on MURASHIGE and SKOOG (1962) medium (MS) containing 5.0 µM zeatin (Sigma, St. Louis, MO, USA), 2.46 µM IBA (Sigma) and solidified with 8 g l^{-1} agar (Unilab, Saarchem, South Africa). Cultures were kept under a continuous photoperiod in a growth room fitted with cool white-fluorescent lamps (Osram L75W/20X, USA), providing a PPFD of 35 ± 2 μmol m^{-2} s^{-1}, at 25 ± 2 ℃, and a relative air humidity of approximately 60%. Shoots from *in vitro* grown plants were used as initial explants in the present study.

2.2.2 Experimental design and culture conditions

Three concentrations of NH_4^+ ions in the medium (10.3, 20.6 and 61.8 mM) and three types of CKs [zeatin, BA (Sigma) and TDZ (Sigma)], applied at three concentrations (0, 5 and 15 µM) were tested in multifactorial combinations. The basal medium consisted of MS salts and vitamins supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma) and 2.46 µM IBA. The pH of all media was adjusted to 5.8 with 1 M KOH or 1 M HCl and 8 g l^{-1} agar was added prior to autoclaving at 121 ℃ at 1 05 kPa for 20 min. Explants, 3 - 4 cm long and with five to six leaves were obtained from *in vitro* grown plantlets as specified above. They were cultured in 250 ml tissue culture flasks, each containing 50 ml of medium and closed with polypropylene screw caps. Six explants were planted per flask. Altogether there were 27 treatment combinations, and each treatment comprised 18 explants. The cultures were incubated at 25 ± 2 ℃ under a continuous photoperi od (see above).

2.2.3 Data collection and statistical analysis

At the end of the 8-week culture period the number of shoots per explant (multiplication rate) was recorded, and the newly-formed shoots were categorized as

normal shoots (NS) or hyperhydric shoots (HS), according to their external appearance (ZIV, 1991). HS had thicker, translucent and water-logged leaves compared to NS, whose leaves showed no abnormality. Hyperhydricity (%) was calculated based on the above information: number of HS per explant / total number of shoots per explant × 100.

The experimental design included three levels of NH_4^+ ions, three types of CKs and three CK concentrations. The hyperhydricity (%) data were transformed into arcsine square roots prior to statistical analysis. The data on multiplication rate and hyperhydricity were analysed by multifactorial analysis of variance (ANOVA) using a completely randomised block design. GenStat 9 (Release 9.1) software (McCONWAY *et al.*, 1999) was used to perform all analyses. Least significant differences (LSD) test was used as a post hoc comparison of means at $P \leq 0.05$.

2.3 Results

Analysis of variance indicated that all factors tested: NH_4^+ concentration, CK type and CK concentration, and all interactions between them had significant effects on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *A. polyphylla* after 8 weeks in culture (Table 2.1). However, to some extent, these effects could be due to: (a) the relatively low multiplication rate on media supplemented with no cytokinins (0 µM) or with TDZ (Table 2.2), and (b) the low occurrence of hyperhydricity on media with 10.3 mM NH_4^+ or media containing TDZ (Figure 2.4).

Table 2.1: Statistical significance of the effect of NH_4^+ concentration and cytokinin type and concentration on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *A. polyphylla* after 8 weeks in culture. NH_4^+ concentrations (mM): 10.3, 20.6 and 61.8; cytokinin types: zeatin, BA and TDZ, applied at the following concentrations (µM): 0, 5 and 15

	F-probability level	
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)
NH_4^+ concentration	< 0.001 *	< 0.001 *
CK type	< 0.001 *	< 0.001 *
CK concentration	< 0.001 *	< 0.001 *
NH_4^+ conc. × CK type	< 0.001 *	< 0.001 *
NH_4^+ conc. × CK conc.	< 0.001 *	< 0.001 *
CK type × CK conc.	< 0.001 *	< 0.001 *
NH_4^+ conc. × CK type × CK conc.	< 0.001 *	0.050 *

* - indicates significant treatment effect

F-probability level ≤ 0.05

The response of the explants to different media compositions was very diverse, as well as the morphology of the new shoots produced (Figures 2.1, 2.2 and 2.3). Regeneration occurred in all treatments tested, including the media lacking exogenous cytokinins (0 µM) (Table 2.2). On media with no cytokinins an average of 2.5 shoots per explant were produced. They had a healthy appearance and dark green leaves (Figure 2.1, A). The initial explants and some of the new shoots formed roots, which is a typical response of many herbaceous species when placed on a hormone-free medium. This was also previously reported for *A. polyphylla* by CHUKWUJEKWU *et al.* (2002). However, the rooting was inhibited with the increase of NH_4^+ concentration in the media (Figure 2.1, A). Spontaneous HS also appeared on cytokinin-free media (Figure 2.4), and they appeared exactly the same as regular HS. Browning of the media was not observed.

Table 2.2: Effect of NH_4^+ concentration and exogenous cytokinin type and concentration on the multiplication rate (number of shoots per explant) of *Aloe polyphylla* after 8 weeks in culture

		Multiplication rate (shoots / explant) [a]		
		Cytokinin type		
NH_4^+ (mM)	CK conc. (µM)	Zeatin	BA	TDZ
10.3	0.0	3.2 i	2.4 i	3.3 i
	5.0	24.0 c	32.1 b	9.3 gh
	15.0	40.6 a	42.7 a	3.5 i
20.6	0.0	3.1 i	3.2 i	2.7 i
	5.0	45.2 a	42.8 a	2.3 i
	15.0	25.3 c	20.3 cd	2.0 i
61.8	0.0	1.7 i	1.2 i	1.6 i
	5.0	14.6 ef	18.5 de	1.4 i
	15.0	11.8 fg	6.1 hi	0.8 i

[a] Means followed by common letters (across all the data) are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test. LSD (5.3) was calculated for the interaction between NH_4^+ concentration, CK type and CK concentration

When the culture medium was supplemented with exogenous zeatin or BA, both axillary and adventitious shoots were formed, with the latter originating from the base of the initial explant. New vigorous shoots of high quality were produced on media with 10.3 mM NH_4^+ and 5 µM zeatin or BA, yielding an average of 24 and 32 shoots per explant, respectively (Table 2.2). Very low hyperhydricity (1.4 – 3.0%) was observed (Figure 2.4, A). Increasing the concentration of zeatin or BA to 15 µM, resulted in an increase of the multiplication rate to 41 and 43 shoots per explant, correspondingly, and a slight non-significant increase of hyperhydricity. However, the newly-formed shoots were fragile, stunted, often etiolated, and frequently formed clusters (particularly when BA was used) (Figures 2.1, C and 2.2, C). Similar results in terms of number of shoots per explant and occurrence of hyperhydricity were

observed when NH_4^+ ions were increased to 20.6 mM and zeatin or BA used at 5 µM (Table 2.2, Figure 2.4, B). The new shoots were visibly longer and displayed a healthier appearance, despite some of them being etiolated because of high multiplication rate. Increasing the concentration of zeatin or BA to 15 µM resulted in an approximately 50% decrease of the multiplication rate, and 2.5- and 4.3-fold increase of hyperhydricity, respectively. The quality of the shoots was also compromised – they were brittle and delicate and had a pale green colour (Figures 2.1, C and 2.2, C). Increasing the concentration of NH_4^+ in the media to 61.8 mM reduced further the number of shoots obtained, and this outcome was even more pronounced when the concentration of zeatin or BA was raised to 15 µM (Table 2.2). The occurrence of hyperhydricity escalated under these conditions, reaching a maximum of 76% on media with 15 µM BA (Figure 2.4, C). The signs of poor quality were largely manifested in shoots from these treatments, characteristically being low in vigour, soft and fragile, with a yellowish - pale green colour. Some of the new shoots and even initial explants developed necrosis and died. Rooting occurred only on media with low concentrations of NH_4^+ (10.3 and 20.6 mM) and zeatin or BA (5 µM). Excretion of phenolic compounds into the medium (browning) was observed in all treatments; however, it became more intensive when the concentrations of zeatin or BA and/or NH_4^+ in the media were high.

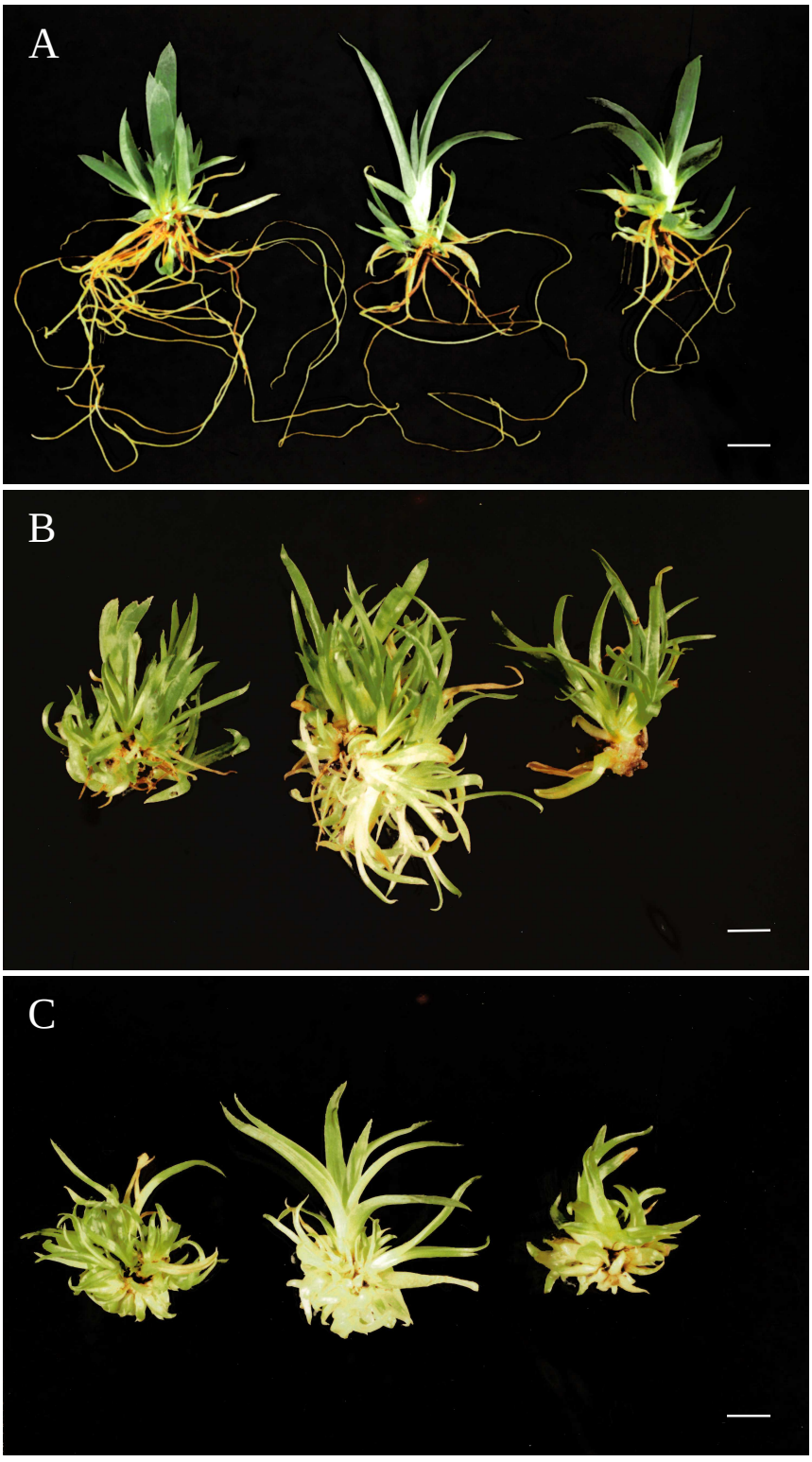

Figure 2.1: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 5 µM (B) or 15 µM (C) zeatin and NH_4^+ ions at three concentrations (from left to right): 10.3, 20.6 and 61.8 mM. Bar = 10 mm.

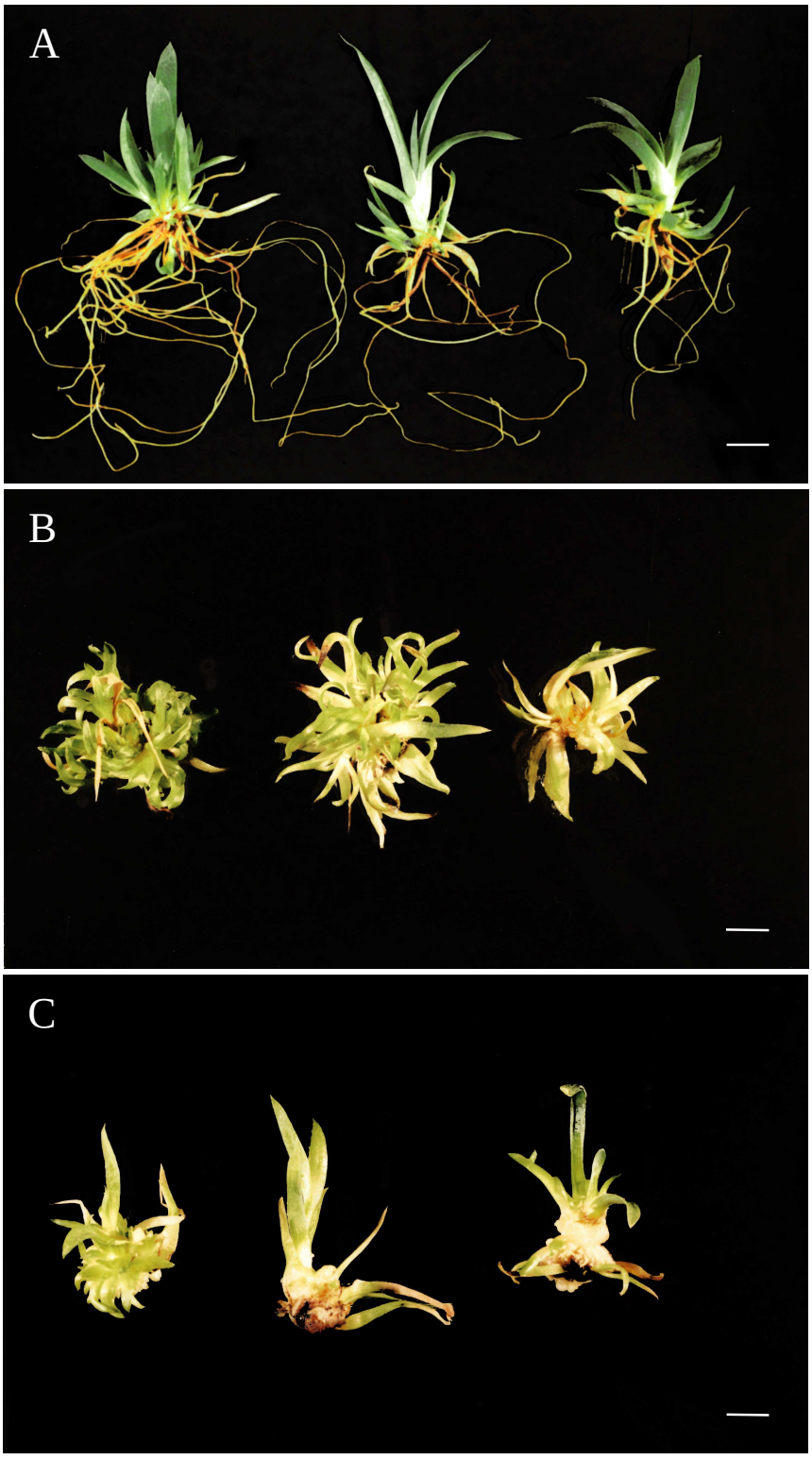

Figure 2.2: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 5 µM (B) or 15 µM (C) BA and NH_4^+ ions at three concentrations (from left to right): 10.3, 20.6 and 61.8 mM. Bar = 10 mm.

The addition of TDZ as an exogenous cytokinin to the basal medium resulted in a very low multiplication rate (Table 2.2). Increasing the concentrations of TDZ and NH_4^+ ions decreased the number of shoots produced per explant; however, with one exception, these differences were non-significant. Plants grown on media with TDZ displayed very distinctive morphological features (Figure 2.3, B and C). After 8 weeks in culture, the initial explants had swollen bases (approx. d = 10 – 15 mm) and formed many buds, the growth of which was inhibited (length = 0.5 - 1.0 mm) and they did not develop into shoots. Many explants produced also watery, brown in colour, non-organogenic callus at their bases. In almost all treatments with TDZ, the meristematic area and the youngest leaves of the initial explants and often of the newly-formed shoots became necrotic and died. The new shoots and buds were very brittle. The occurrence of hyperhydricity was not very high, as many shoots and buds did not show a translucent appearance (Figure 2.4). However, as their fate was not investigated further, it is uncertain if they had the potential to develop into normal plantlets, and hence to make the use of TDZ beneficial in terms of achieving a low hyperhydricity. No rooting occurred on media supplemented with TDZ. High excretion of phenolic compounds into the media was also typical.

To summarize, there was a clear tendency of the effect of the investigated factors regarding the occurrence of hyperhydricity: increasing the concentration of NH_4^+ ions and cytokinin concentration (in the case of zeatin or BA) led to a proportional increase of hyperhydricity (Figure 2.4). However, the multiplication rate was influenced in a more complex way, depending to a big extent on the cytokinin concentration applied (Table 2.2).

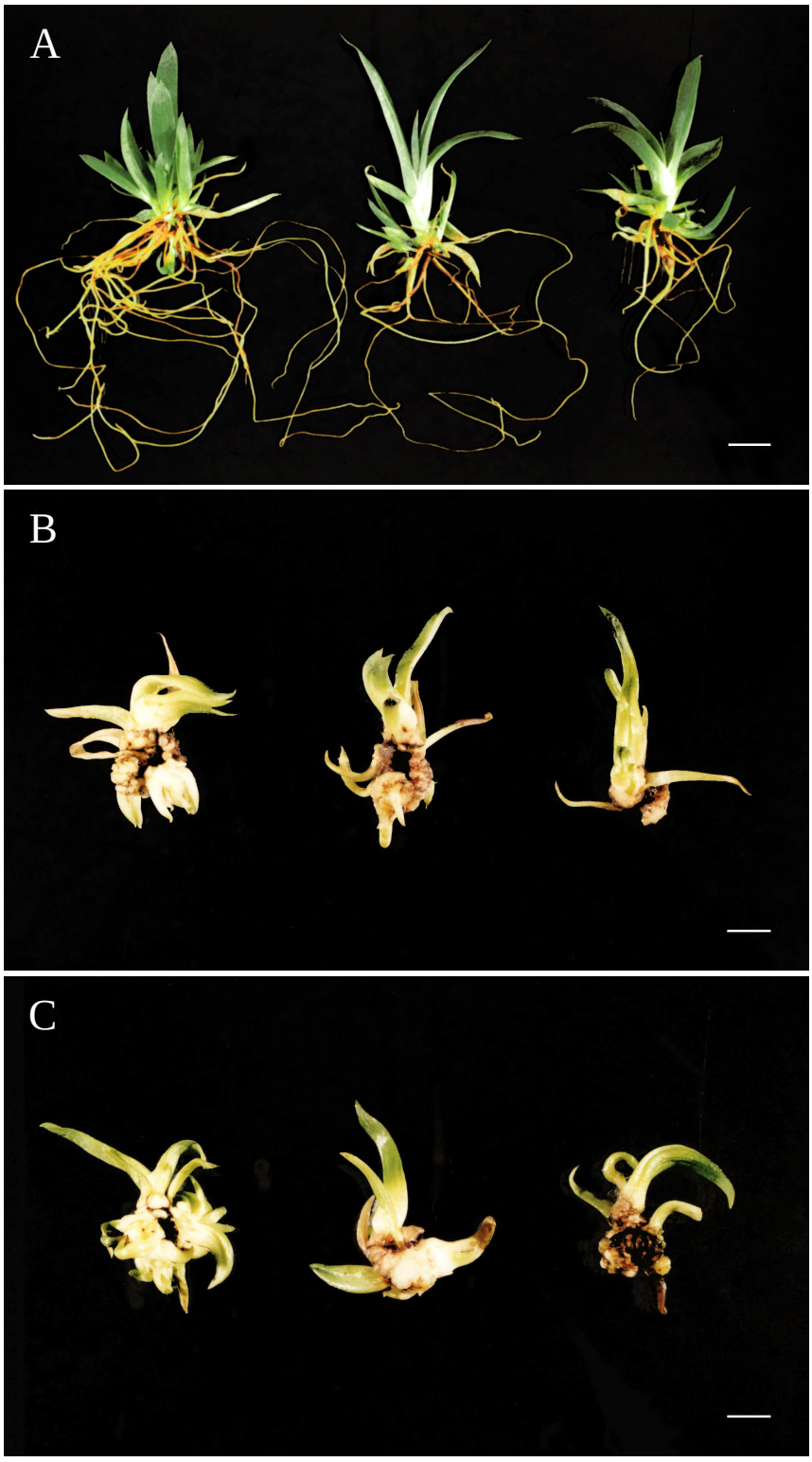

Figure 2.3: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 5 µM (B) or 15 µM (C) TDZ and NH_4^+ ions at three concentrations (from left to right): 10.3, 20.6 and 61.8 mM. Bar = 10 mm.

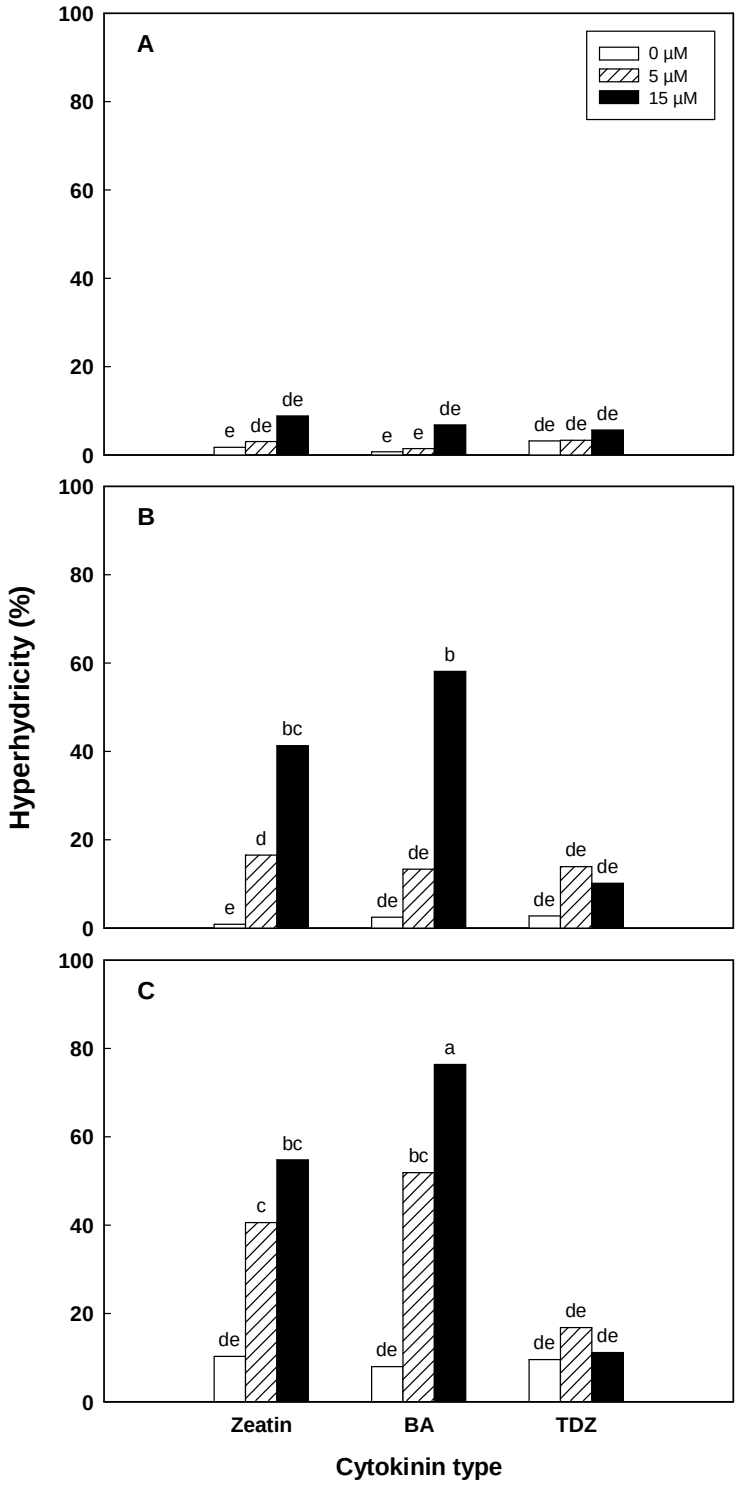

Figure 2.4: Hyperhydricity (%) of *A. polyphylla* shoots obtained after 8 weeks of culture on media supplemented with 10.3 mM (A), 20.6 mM (B) or 61.8 mM (C) NH_4^+ and zeatin, BA or TDZ, applied at 0, 5 or 15 µM. Bars with common letters (across the three graphs – A, B and C) are not significantly different at $P \leq 0.05$, according to LSD test. LSD (14.6) was calculated for the interaction between NH_4^+ concentration, CK type and CK concentration.

2.4 Discussion

2.4.1 Effect of exogenous cytokinins on multiplication rate and hyperhydricity of Aloe polyphylla

2.4.1.1 Effect of BA and zeatin

The addition of cytokinins, which regulate cell division and differentiation (JACQMARD *et al.*, 1994; WERNER *et al.*, 2001), to the culture medium is essential for shoot regeneration *in vitro* (GEORGE, 1993). It has been shown that BA is readily taken up by the shoots from the medium (NORDSTRÖM and ELIASSON, 1986; FEITO *et al.*, 1995; AUER *et al.*, 1999) and rapidly metabolised into a number of derivatives (VAN STADEN and CROUCH, 1996). An earlier report from our laboratory (IVANOVA *et al.*, 2006) indicated that the endogenous cytokinin pool of *A. polyphylla* shoots grown on media with BA for 8 weeks was comprised by over 90% of BA-type cytokinins, while in shoots obtained from zeatin-supplemented media it consisted of isoprenoid and isoprenoid-derived cytokinins. The present study showed that increasing the concentration of BA or zeatin resulted in an increase of the multiplication rate but only on media with 10.3 mM NH_4^+ ions (Table 2.2). With elevating the amount of ammonium in the medium, an inverse correlation between cytokinin concentration and multiplication rate was observed. CHUKWUJEKWU *et al.* (2002) also reported that on MS medium (containing 20.6 mM NH_4^+) high levels of BA or zeatin did not improve shoot multiplication of *A. polyphylla*. Raising the concentration of exogenous BA and zeatin in the MS medium (20.6 mM NH_4^+) had a positive effect on the accumulation of endogenous cytokinins in *A. polyphylla* shoots (IVANOVA *et al.*, 2006). However, this did not translate into a high multiplication rate.

On the one hand, it appears that the decisive factor in inducing hyperhydricity on media with 20.6 and 61.8 mM NH_4^+ ions is the addition of BA or zeatin (Figure 2.4, B and C). Increasing the concentration of ammonium ions in media without the presence of cytokinins did not cause a significant increase in the incidence of hyperhydricity (Figure 2.4). Others have also identified that the hyperhydric state depends on cytokinins as the primary factor (GASPAR *et al.*, 1987; LESHEM *et al.*, 1988; FRICK, 1991; PHAN, 1991). Furthermore, we observed that the percentage of

hyperhydricity was proportional to the concentration of BA or zeatin, in that, the highest BA or zeatin concentration resulted in the highest occurrence of HS (Figure 2.4, B and C). BA has been especially noted to increase hyperhydricity when used at high concentrations (DEBERGH, 1983; DENCSO, 1987; KATAEVA *et al.*, 1991; WILLIAMS and TAJI, 1991; HOSOKI and TAHARA, 1993; TSAY, 1998). Similar dose-dependent elevation in hyperhydricity was observed for zeatin as well (JACOBONI and STANDARDI, 1987; IVANOVA *et al.*, 2006). On the other hand, adding BA or zeatin to media containing low NH_4^+ (10.3 mM) did not induce hyperhydricity (it was not significantly different from spontaneous hyperhydricity, which occurred on cytokinin-free media; Figure 2.4, A). This result suggests that BA and zeatin are liable to induce the glassy shoot syndrome in *A. polyphylla*, only when other factors in the culture system are not optimal (i.e. high NH_4^+ ions concentration). These hyperhydricity-inducing tendencies of cytokinins, coupled with hyperhydricity-favouring culture conditions have been well documented in the literature (BORNMAN and VOGELMANN, 1984; PAQUES and BOXUS, 1987b; WILLIAMS and TAJI, 1991; LI *et al.*, 1997; THOMAS *et al.*, 2000).

2.4.1.2 Effect of TDZ

In the present study we compared the effect of three types of cytokinins on the multiplication rate and hyperhydricity of *in vitro* regenerants of *A. polyphylla*, namely BA – an aromatic cytokinin, zeatin – an isoprenoid cytokinin, and TDZ – a synthetic diphenylurea derivative. While BA and zeatin affected the above parameters in a similar manner, the effect of TDZ differed markedly. Characteristically, TDZ is a non-expensive, very potent cytokinin, the high activity of which may be due partly to its extreme stability in plant tissues (MOK *et al.*, 2000). The mode of action of TDZ may be indirect, by regulating endogenous cytokinin biosynthesis or metabolism, or direct, through its own biological activity (MOK and MOK, 1985), since common receptors for both adenine type and phenylurea cytokinins have been discovered (YAMADA *et al.*, 2001).

Although it is used predominantly for tissue culture of woody plants (HUETTEMAN and PREECE, 1993, and references therein), positive responses of herbaceous species to TDZ have also been reported (MEYER and VAN STADEN, 1991;

NAKANO *et al.*, 1994; McCARTAN and VAN STADEN, 1998). The addition of TDZ to the basal medium resulted in a very low multiplication rate compared to BA and zeatin (Table 2.2). Extremely low concentrations of TDZ (in the range of pM and nM) stimulate axillary shoot proliferation of many species (see HUETTEMAN and PREECE, 1993 for a review). Therefore, as noted by these authors, direct comparisons at equimolar concentrations between TDZ and amino purine cytokinins (for which activity is in the range of 1 – 10 µM) are complicated and difficult. This range with TDZ often results in callus formation and cessation of shoot growth. The use of high TDZ levels (5 and 15 µM) is one possible explanation for the results observed here – low multiplication rate, inhibited bud growth, the swollen base of initial explants and frequent callus formation. On the other hand, many species have responded to similar TDZ concentrations with the formation of both axillary and adventitious shoots (see HUETTEMAN and PREECE, 1993 for a review). The use of 5 µM TDZ as the sole growth regulator was detrimental to shoot explants of *Aloe barbadensis*, however the addition of 5 µM IBA together with 5 µM TDZ resulted in an average production of 19 buds per explant (MEYER and VAN STADEN, 1991). Taken together, it appears that the response to TDZ depends largely on the concentration used, the plant species and the balance of plant growth regulators in the medium. Therefore, further investigations are needed to elucidate the potential of this cytokinin for use in the micropropagation of *A. polyphylla*.

Although the newly-formed shoots and buds on media with TDZ were very fragile and looked abnormal, compared to shoots obtained on media with BA or zeatin, the majority of them did not display a glassy, watery appearance, and were not considered as hyperhydric. Thus, the percentage of hyperhydricity on media with TDZ was very low (Figure 2.4). However, given the low multiplication rate, inhibited bud growth, and the poor quality of the new shoots and buds, TDZ does not appear to be a suitable cytokinin for mass propagation of *A. polyphylla*.

2.4.2 Effect of NH_4^+ ion concentration on multiplication rate and hyperhydricity of Aloe polyphylla

The results presented here have shown that the morphology, multiplication rate and occurrence of hyperhydricity of *in vitro* regenerated shoots of *A. polyphylla* are greatly

influenced by the amount of NH_4^+ ions in the culture medium. Characteristically, MS media has a high concentration of macronutrients and is particularly rich in NH_4^+ ions (20.6 mM). At this concentration, NH_4^+ ions have been reported to induce hyperhydricity in *Salix babylonica* L. (BEAUCHESNE, 1981; DAGUIN and LETOUZÉ, 1986), *Prunus avium* (RIFFAUD and CORNU, 1981), *Castanea sativa* Mill. (VIEITEZ *et al.*, 1985), *Dianthus caryophyllus* L. (ZIV *et al.*, 1987) and *Amelanchier arborea* Michx.f. (grown on woody plant medium containing 20 mM NH_4NO_3; BRAND, 1993). The phenomenon of hyperhydricity was inversely correlated with the degree of lignification of the tissues, which decreased with increasing hyperhydricity (VIEITEZ *et al.*, 1985; LETOUZÉ and DAGUIN, 1987). Our results showed that elevating the levels of NH_4^+ ions, in the absence of cytokinins in the medium, could not induce hyperhydricity (Figure 2.4). However, in the presence of cytokinins, normal concentrations of NH_4^+ ions in the MS medium – 20.6 mM and higher, stimulated the induction of hyperhydricity (Figure 2.4, B and C). Increasing the NH_4NO_3 concentration above the standard MS level produced much softer gels when gelrite was used (HUANG *et al.*, 1995), and thus making substances, which are responsible for the induction of hyperhydricty, such as water, cytokinins and NH_4^+ ions (see below), readily available to the explants. Lowering the amount of NH_4^+ ions to a half of its value in the MS medium eliminated hyperhydricity (Figure 2.4, A). Similarly, reduction in the occurrence of HS was observed in cultures of *Salix babylonica* L. (BEAUCHESNE, 1981), *Prunus avium* (RIFFAUD and CORNU, 1981) and *Castanea sativa* Mill. (VIEITEZ *et al.*, 1985) when grown on media containing a half or a third of the original concentration of NH_4NO_3 in MS medium.

2.4.3 Possible causes of hyperhydricity

One possible explanation of the mechanism by which NH_4^+ ions affect the water availability, and thus play a role in the induction of hyperhydricity has been suggested in the literature. Deficiency of lignin and cellulose biosynthesis may results from a fall in the C/N ratio after increased assimilation of N. Ammonium ions are normally taken up more rapidly from the plant culture medium than other nitrogen sources such as NO_3^-. This could result in plant tissues coming to possess physiologically damaging endogenous concentrations. Such excess of NH_4^+ may induce a detoxification process (GIVAN, 1979), which diverts carbohydrates away from the metabolic

pathways leading to the synthesis of lignin and cellulose, by increasing the activity of glutamate dehydrogenase enzymes. The sugars are then utilised in the production of organic nitrogen compounds, i.e. amino acids (MIFLIN and LEA, 1980). Glutamate dehydrogenase activity has been reported to be higher in hyperhydric tissues (DAGUIN and LETOUZÉ, 1986). As a result of the above described changes, the process of de-amination of L-phenylalanine ceases, leading in turn to a deficiency of cinnamic acid and hence *p*-coumaric acid, from which lignin is made. Hyperhydric tissues have been noted to have lower activity of phenylalanine ammonia-lyase enzyme (KEVERS and GASPAR, 1985a; LETOUZÉ and DAGUIN, 1987). A number of histological studies have shown a reduced lignification in hyperhydric plants (LESHEM, 1983a; VIEITEZ *et al.*, 1985; OLMOS and HELLÍN, 1998; FONTES *et al.*, 1999; PICOLI *et al.*, 2001). This deficiency would tend to reduce wall pressure and thus favour increased water uptake, which could explain the hyperhydric state (KEVERS *et al.*, 1984).

The C/N ratio has also been associated with the levels of phenolic compounds (SHULER, 1981). Decreased phenol levels in hyperhydric plants (KEVERS *et al.*, 1984; VIEITEZ *et al.*, 1985) could be explained with the decrease in the C/N ratio. However, PHAN and LETOUZÉ (1983) found higher levels of phenolics in HS of *Prunus avium* than in NS. But since the hydroxycinnamate CoA ligase activity was lower in HS, these higher amounts of phenolics could be a result of their reduced utilisation for the biosynthesis of lignins or flavonoids.

GASPAR *et al.* (1987) have proposed a putative role of ethylene in the induction of hyperhydricity. According to their hypothesis, an excess of ethylene (as a response to stress conditions, e.g. high NH_4^+ ions or cytokinin concentration) in the culture atmosphere decreased the activities of phenylalanine ammonia-lyase and acid peroxidases and thus hindered lignification.

2.5 Conclusions and Recommendations

Taken together, our results showed that in tissue culture of *A. polyphylla* BA and zeatin are able to induce hyperhydricity, in a concentration-dependent manner, only when other factors in the culture system are not optimised, i.e. high NH_4^+ ions

concentration. For high multiplication rate and low incidence of hyperhydricty we suggest the use of low NH_4^+ ions in the medium (10.3 mM) combined with low BA concentration (5 µM), which results in the production of shoots of high quality. Although in the present investigation the use of TDZ resulted in the production of poor quality shoots, additional research is needed to fully elucidate its potential for use in mass propagation of *A. polyphylla*. In our study we elevated the concentration of NH_4^+ in the medium by the addition of NH_4NO_3, and thus, NO_3^- ions were simultaneously elevated along with the NH_4^+ ions. However, this makes it difficult to ascertain if the results obtained were associated with the NH_4^+ or NO_3^- ions. With the intention of clarifying this, we pursued further investigations, the results of which are presented in the next chapter.

Chapter 3

NITROGEN SOURCE AND CONCENTRATION, AND NH_4^+ : NO_3^- RATIO INFLUENCE HYPERHYDRICITY AND SHOOT REGENERATION IN TISSUE CULTURED *ALOE POLYPHYLLA*

3.1 Introduction

Since DEBERGH *et al*. (1981) first reported and defined as such the phenomenon of 'vitrification', a term later substituted with hyperhydricity (DEBERGH *et al.*, 1992), much comprehensive research has been done and a number of reviews have been written on the subject (GASPAR *et al.*, 1987; PÂQUES and BOXUS, 1987a; GASPAR, 1991; PÂQUES, 1991; ZIV, 1991; DEBERGH *et al.*, 1992; GEORGE, 1993). During the process of hyperhydricity, shoots experience problems with differentiation (KEVERS *et al.*, 2004) and undergo detrimental changes at anatomical, physiological and morphological levels (see GASPAR, 1991; ZIV, 1991 for reviews). Consequently, the capacity of hyperhydric shoots (HS) for further survival in the *in vitro* environment or upon transfer to external conditions could be severely reduced. Up to a 60% loss in the production during commercial micropropagation can occur due to hyperhydricity (PÂQUES, 1991). The complexity of this phenomenon is reflected by the fact that it is difficult to distinguish which of the many changes in the HS have engendered the underdifferentiated state and which are the consequences of it. Some of the changes, typically associated with hyperhydricity, include impaired chloroplast organization (WETZSTEIN and SOMMER, 1982; JONES *et al.*, 1993; LOURO *et al.*, 1999), a reduced amount of chlorophyll (DONNELLY and VIDAVER, 1984; JONES *et al.*, 1993; SAHER *et al.*, 2004), large intercellular spaces in the mesophyll cell layer (PÂQUES and BOXUS, 1987b; WERKER and LESHEM, 1987; PICOLI *et al.*, 2001), deposition of little or no epicuticular wax (ZIV *et al.*, 1983; ZIV and ARIEL, 1994; OLMOS and HELLÍN, 1998) and an increase in endogenous cytokinin levels (KATAEVA *et al.*, 1991; DANTAS DE OLIVEIRA *et al.*, 1997; VANDEMOORTELE *et al.*, 2001; IVANOVA *et al.*, 2006).

Interestingly, a study with a recessive *Arabidopsis* mutant, designated *cristal* (*cri1*), which displayed the above mentioned abnormalities reminiscent of hyperhydric symptoms, has shown that none of them are sufficient by themselves to confer the vitreous phenotype (DELARUE *et al.*, 1997). In *Aloe polyphylla* too, the increase in the endogenous level of cytokinins in HS could not alone account for the induction of hyperhydricity (discussed in Chapter 6; IVANOVA *et al.*, 2006). These findings are indicative of the multifactor nature of this elusive phenomenon.

It appears that the metabolic perturbations in the *in vitro* regenerants, leading to hyperhydricity, are induced by the combined action of several physical (e.g. gel solidity, type of gelling agent, relative humidity) and/or chemical (e.g. type and level of plant growth regulators, concentration of mineral nutrients, ethylene and CO_2 concentrations in the gaseous phase) factors of the culture environment (see GASPAR, 1991; ZIV, 1991; DEBERGH *et al.*, 1992 for a complete list). This complexity prevents the identification of processes which are causal to hyperhydricity as opposed to processes which only affect its occurrence in a quantitative manner.

We have shown that the incidence of hyperhydricity in micropropagated shoots of *Aloe polyphylla*, the species in the present investigation, is affected by the synergistic action of cytokinin concentrations and NH_4^+ ion levels (discussed in Chapter 2; IVANOVA and VAN STADEN, 2008). However, in this study and some previous reports (VIEITEZ *et al.*, 1985; DAGUIN and LETOUZÉ, 1986; BRAND, 1993), NH_4^+ ions were supplied in the form of NH_4NO_3, thus elevating the concentration of NH_4NO_3 in the media resulted in a simultaneous raise of NH_4^+ and NO_3^- ions. This made it difficult to determine exactly which of both ions were associated with the increase in hyperhydricity. Elevating the amount of NH_4NO_3 led also to an overall increase of total nitrogen in the media, hence media with concentrations of 10, 20 and 60 mM NH_4^+ contained 40, 60 and 140 mM of total nitrogen, respectively (IVANOVA and VAN STADEN, 2008). Excessive nitrogen content too, could not be ruled out as a possible cause for the higher degree of hyperhydricity.

The tissue culture media used for regeneration of *A. polyphylla* shoots (ABRIE and VAN STADEN, 2001; CHUKWUJEKWU *et al.*, 2002; IVANOVA *et al.*, 2006; BAIRU *et al.*, 2007; IVANOVA and VAN STADEN, 2008) are based on the mineral nutrient

composition formulated by MURASHIGE and SKOOG (1962), where typically the nitrogen pool of 60 mM is comprised of inorganic ammonium and nitrate at a ratio of $20NH_4^+ : 40NO_3^-$ (mM). However, no study was conducted to provide a physiological basis for using this nitrogen concentration or ratio for the micropropagation of this species. Noticeable influences of the absolute and relative amounts of nitrate and ammonium on the growth and morphogenesis of plant tissue cultures have been reported for a number of *in vitro* systems (WETHERELL and DOUGALL, 1976; GRIMES and HODGES, 1990; EVANS, 1993; TSAI and SAUNDERS, 1999; RAMAGE and WILLIAMS, 2002; WOODWARD *et al.*, 2006).

In the experiments described in this study, the influence of inorganic ammonium and nitrate and organic glutamine as sole nitrogen sources as well as the ratio of $NH_4^+ : NO_3^-$ were evaluated with special emphasis placed on hyperhydricity occurrence and shoot organogenesis of tissue cultured *Aloe polyphylla*. The nitrogen requirements for low incidence of hyperhydricity, high regeneration rate and production of good quality shoots have been clarified.

3.2 Materials and Methods

3.2.1 Plant material

Tissue culture plantlets of *Aloe polyphylla* were initially micropropagated using successfully germinated seedlings (ABRIE and VAN STADEN, 2001). The culture was subsequently maintained and multiplied by subculturing the shoots at an interval of eight weeks. They were grown on basal MURASHIGE and SKOOG (1962) medium (MS) supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma, St. Louis, MO, USA), 5.0 µM zeatin (Sigma) and 2.46 µM IBA (Sigma). The pH of the medium was adjusted to 5.8 with 1 M KOH or 1 M HCl and 8 g l^{-1} agar (Unilab, Saarchem, South Africa) was added prior to autoclaving at 121 ℃ at 105 kPa for 20 min. The cultures were maintained under a continuous photoperiod in a growth room fitted with cool white-fluorescent lamps (Osram L75W/20X, USA), providing a light intensity of 35 ± 2 μmol m^{-2} s^{-1}, at 25 ± 2 ℃, and a relative air humidity of approximately 60%. Shoots from *in vitro* grown plants were used as initial explants in the present experiments.

3.2.2 Experimental designs

In the first experiment, three sources of nitrogen in the culture media (NH_4^+ only, NO_3^- only, or a combination of both NH_4^+ and NO_3^-), applied at four concentrations (0, 30, 60 and 90 mM) were tested in factorial combinations. The MS basal medium was modified in one of the following ways:

(i) To achieve the presence of NH_4^+ ions only, NH_4NO_3 and KNO_3 salts were excluded from the MS basal medium and substituted with NH_4Cl.

(ii) To ensure the presence of NO_3^- ions only in the medium, NH_4NO_3 salt was kept out of the basal medium and nitrogen was supplied only in the form of KNO_3.

(iii) Nitrogen-free medium was ascertained by leaving out of the basal medium the salts containing any form of nitrogen, i.e. NH_4NO_3 and KNO_3.

In a separate experiment, glutamine, as an organic source of nitrogen, was tested at four concentrations: 0, 0.5, 1.5 and 5.0 g l^{-1}. The MS basal medium did not contain any form of inorganic nitrogen (NH_4NO_3 and KNO_3 salts were eliminated).

In the final experiment, we modulated the ratio of NH_4^+ : NO_3^- ions, while keeping the total concentration of nitrogen constant at 60 mM, as in the MS medium. Seven ratios of NH_4^+ : NO_3^- ions (mM) were tested – 0 : 60, 10 : 50, 20 : 40, 30 : 30, 40 : 20, 50 : 10 and 60 : 0. The ionic compositions of macronutrients for the various media preparations were calculated using a program in BASIC: ION-1, listed in Appendix 1 of Plant Culture Media (GEORGE *et al.*, 1988), and are shown in Table 3.1. Care was taken to ensure that the sum of the positive ions in each of the mixtures equals the sum of the negative ions.

Table 3.1: Ionic composition of macronutrients for seven media formulations with different NH_4^+ : NO_3^- ion ratios

	Ionic concentration of macronutrients (meq l^{-1}) [a]						
	NH_4^+ : NO_3^- ratio (mM)						
Ion	0 : 60	10 : 50	20 : 40	30 : 30	40 : 20	50 : 10	60 : 0
NO_3^-	60.00	50.00	39.40	30.00	20.00	10.00	0.00
PO_4^{3-}	3.74	3.74	3.74	3.74	3.74	3.74	3.74
SO_4^{2-}	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Cl^-	1.00	1.00	5.98	19.73	32.73	45.73	65.73
K^+	56.27	36.27	20.04	15.00	8.00	1.00	1.00
Ca^{2+}	5.98	5.98	5.98	5.98	5.98	5.98	5.98
Mg^{2+}	3.00	3.00	3.00	3.00	3.00	3.00	3.00
NH_4^+	0.00	10.00	20.61	30.00	40.00	50.00	60.00
H^+	2.49	2.49	2.49	2.49	2.49	2.49	2.49
Total N	60.00	60.00	60.01	60.00	60.00	60.00	60.00

[a] – Ionic concentrations are given in mequivalence l^{-1}. To obtain the concentrations in mM, divide the meq l^{-1} figure by the valence of the ion

Having calculated the macronutrient ionic composition of each medium, we used another program in BASIC: ION-2, printed also in Appendix 1 of Plant Culture Media (GEORGE *et al.*, 1988), to find the weights of inorganic salts necessary to prepare the media formulations from the ionic equivalents (Table 3.2).

Table 3.2: Weights (mg l^{-1}) of inorganic salts, required to prepare the macronutrient mixtures given in Table 3.1

	Macronutrient composition (mg l^{-1})						
	NH_4^+ : NO_3^- ratio (mM)						
Salt	0 : 60	10 : 50	20 : 40	30 : 30	40 : 20	50 : 10	60 : 0
KNO_3	5461	3439	1900	1170	234	—	—
NH_4NO_3	—	801	1650	1476	1415	801	—
$CaCl_2.2H_2O$	—	—	440	440	440	440	440
$MgSO_4.7H_2O$	370	370	370	370	370	370	370
KH_2PO_4	170	170	170	—	—	—	—
KCl	75	75	—	256	423	75	75
$NH_4H_2PO_4$	—	—	—	143	143	143	143
NH_4Cl	—	—	—	552	1127	2073	3143
$Ca(NO_3)_2.4H_2O$	706	706	—	—	—	—	—

3.2.3 Culture conditions

The basal media were prepared as described above for the various treatments, and supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 5.0 µM zeatin and 2.46 µM IBA. The pH of all media was adjusted to 5.8 and solidified with 8 g l^{-1} agar before autoclaving at 121 ℃ at 105 kPa for 20 min. Explants, with an average length of 3.2 ± 0.5 cm (mean ± SD) and with five to six leaves were obtained from *in vitro* grown plantlets as specified above. They were cultured in 250 ml tissue culture flasks, each containing 50 ml of medium and closed with polypropylene screw caps. Six explants were planted per flask and each treatment comprised 18 explants. Each experiment was repeated at least twice. The cultures were incubated at 25 ± 2 ℃ under a continuous photoperiod (see above).

3.2.4 Data collection and statistical analysis

At the end of the 8-week culture period the number of shoots per explant (multiplication rate) was recorded, and the newly-formed shoots were categorized as normal shoots (NS) or hyperhydric shoots (HS), according to their external

appearance (ZIV, 1991). HS had thicker, translucent and water-logged leaves compared to NS, whose leaves showed no abnormality. Hyperhydricity (%) was calculated based on the above information: number of HS per explant / total number of shoots per explant × 100. To assess further the quality of the shoots produced from the various treatments, the newly-formed shoots were divided according to their length into 2 groups: shoots ≥ 10 mm (big shoots), and shoots < 10 mm (small shoots). The % of big shoots was determined: number of big shoots per explant / total number of shoots per explant × 100. The average length of big shoots for individual treatments was calculated as well.

The hyperhydricity (%) and shoots ≥ 10 mm (%) data were transformed into arcsine square roots prior to statistical analysis. The data on multiplication rate, hyperhydricity and % of big shoots were analysed by one- or two-way analysis of variance (ANOVA) using a completely randomised block design. GenStat 9 (Release 9.1) software was used to perform the analyses. Means were separated by least significant differences (LSD) test at $P \leq 0.05$.

3.3 Results

3.3.1 Effect of nitrogen source and concentration

The number of shoots per explant, occurrence of hyperhydricity and production of big shoots of *A. polyphylla* after 8 weeks in culture were significantly influenced by the interaction between source of nitrogen and concentration (Table 3.3). The morphology of the new shoots produced varied considerably and was associated predominantly with the nitrogen source used (Figure 3.1). Shoot multiplication was observed in all treatments, including when nitrogen was omitted from the culture media (0 mM), where an average of 5.5 shoots per explant were produced and hyperhydricity did not exceed 7% (Figure 3.2, A and B). The new shoots were pale green and very short – on average only 5.3% of the shoots were longer than 10 mm (Figures 3.1 and 3.3, A). Almost no rooting was recorded and the media showed an extensive browning due to the release of phenolic compounds from the explants.

Table 3.3: Statistical significance of the effect of source of nitrogen and concentration in the culture media on the multiplication rate (number of shoots per explant), hyperhydricity (%) and development of shoots longer than 10 mm (%) of *A. polyphylla* after 8 weeks in culture. Source of nitrogen: NH_4^+, NO_3^- and both NH_4^+ + NO_3^- (MS medium), applied at the following concentrations (mM): 0, 30, 60 and 90

	F-probability level		
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)	Big shoots (%)
Nitrogen source	< 0.001 *	< 0.001 *	< 0.001 *
Nitrogen concentration	< 0.001 *	0.092 ns	< 0.001 *
Nitrogen source × conc.	< 0.001 *	< 0.001 *	< 0.001 *

* - indicates significant treatment effect
ns – indicates non-significant treatment effect
F-probability level ≤ 0.05

When NH_4^+ ions were used as the sole source of nitrogen in the culture media, the multiplication rate was very low, irrespectively of the concentration applied (Figure 3.2, A). However, the occurrence of hyperhydricity was the highest across all the treatments in the experiment, reaching a maximum on media with 60 and 90 mM NH_4^+ - 49 and 42%, respectively (Figure 3.2, B). The quality of the newly-formed shoots was poor, with yellowish – pale green colour and frailness being the common features (Figure 3.1, A). No rooting occurred and media browning was intensified with the increase of NH_4^+ in the media. Relatively few new shoots were longer than 10 mm, *ca.* 21% (for 30 and 60 mM NH_4^+) and their average length was approximately 12 mm (Figure 3.3, A and B).

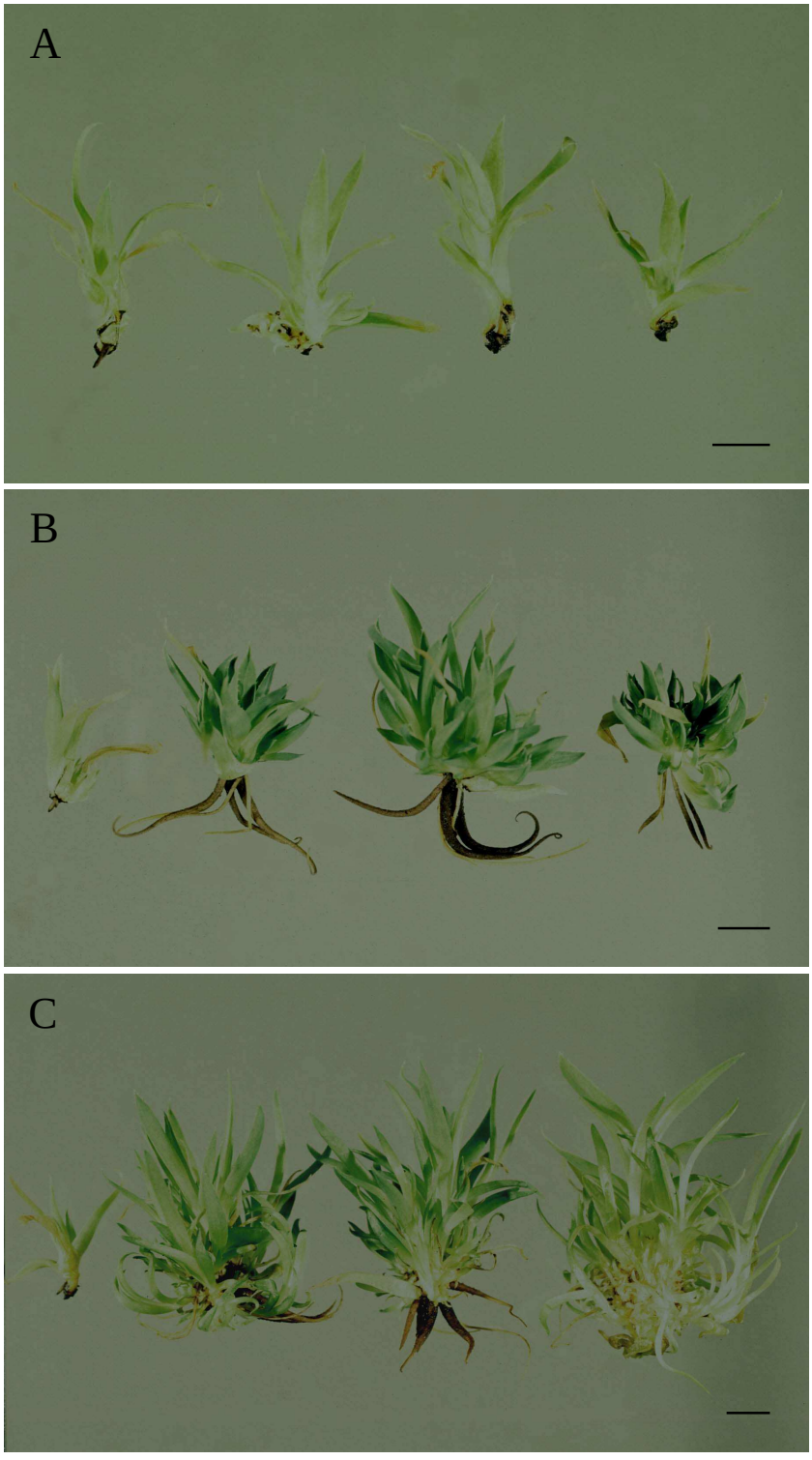

Figure 3.1: Morphology of *A. polyphylla* shoots obtained after 8 weeks in culture on media supplemented with different sources of nitrogen, namely NH_4^+ (A), NO_3^- (B) or both NH_4^+ and NO_3^- (C), at four concentrations (from left to right): 0, 30, 60 and 90 mM. Bar = 10 mm.

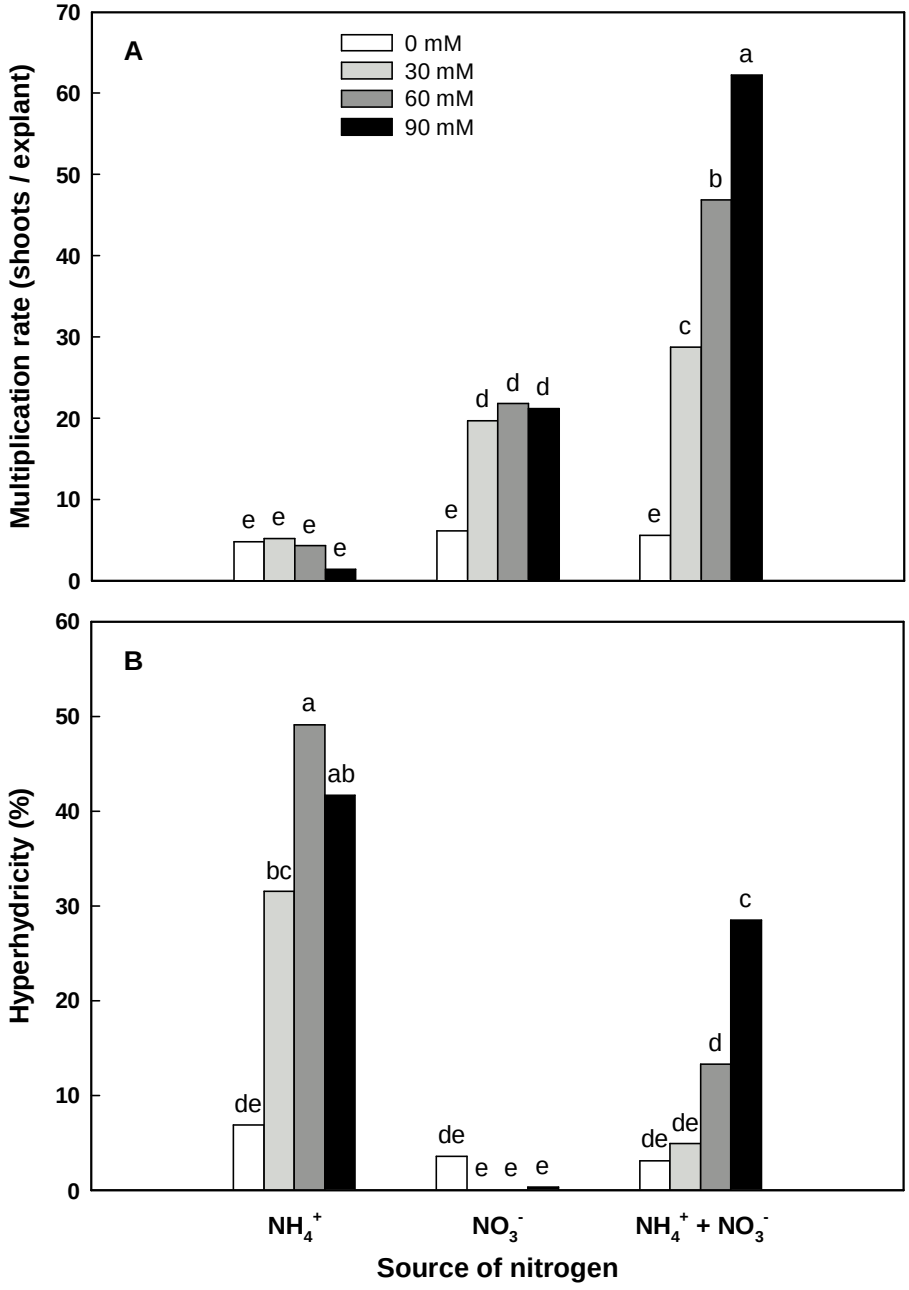

Figure 3.2: Effect of nitrogen source and concentration in the culture media on the multiplication rate (A) and hyperhydricity (B) of *A. polyphylla* shoots after 8 weeks in culture. Bars with common letters are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test. LSD (4.8 – multiplication rate; 12.5 – hyperhydricity) was calculated for the interaction between source of nitrogen and concentration.

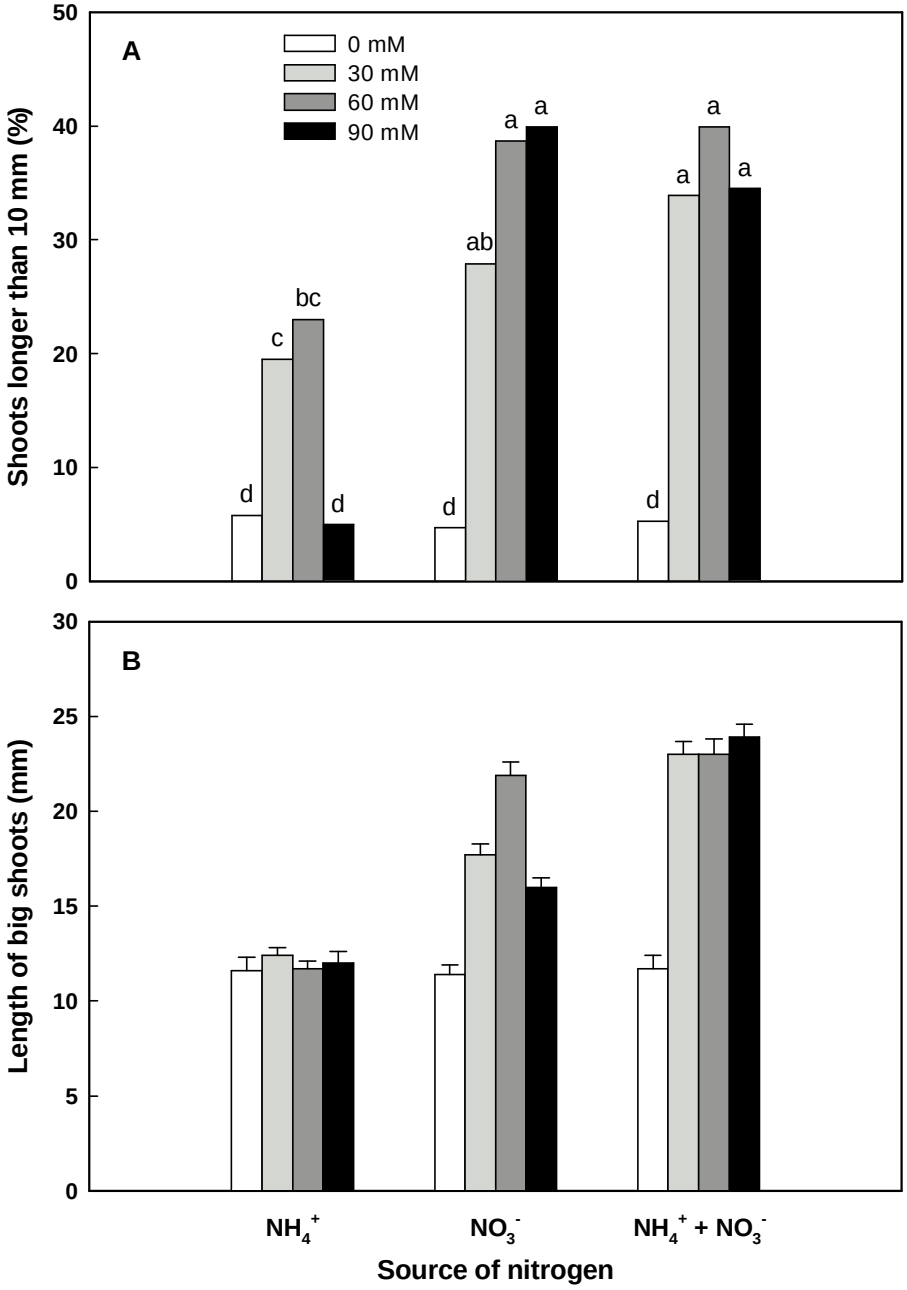

Figure 3.3: Effect of nitrogen source and concentration in the culture media on the development of shoots longer than 10 mm (A) and the average length of shoots ≥ 10 mm (big shoots; B) of *A. polyphylla* after 8 weeks in culture. In (A) bars with common letters are not significantly different at $P \leq 0.05$, according to LSD test (8.9), which was calculated for the interaction between source of nitrogen and concentration. In (B) means are given ± SE (n = 10 – 443).

High-quality new shoots were produced on media supplemented with NO_3^- ions only as the nitrogen source. They had dark green leaves, which often developed soft white teeth on the margins – a sign of vigorousness, and root formation was typical (Figure 3.1, B). Excretion of phenolic compounds into the media was also observed; however, it was less pronounced than when NH_4^+ ions were used. Replacing NH_4^+ with NO_3^- ions in the media resulted in a total elimination of the hyperhydricity and a significant increase in the multiplication rate, which was not NO_3^--concentration dependent and averaged at 21 shoots per explant (Figure 3.2, A and B). Some 28 – 40% of these shoots were longer than 10 mm (Figure 3.3, A) and the highest mean length was recorded for shoots obtained on medium with 60 mM NO_3^- (Figure 3.3, B).

The highest multiplication rate was observed on media containing both NH_4^+ and NO_3^- ions. It increased proportionally with the increase of the total amount of nitrogen in the media, reaching a maximum of 62 shoots per explant (Figure 3.2, A). A similar trend was followed for the hyperhydricity, which was higher with raising the concentration of NH_4^+ and NO_3^- ions (Figure 3.2, B). The newly-formed shoots had a pale green to green colour (Figure 3.1, C) and on average 36% of them had a mean length of 23 mm (Figure 3.3, A and B). The initial explants formed roots frequently and browning of the media was also observed.

3.3.2 Effect of glutamine concentration

Fine new shoots, with green to dark green leaves, were obtained on media with glutamine (Figure 3.4). Increasing the concentration of glutamine in the media resulted in a significant increase in shoot multiplication (Table 3.4; Figure 3.5, A). In contrast, the frequency of hyperhydricity was not affected significantly by the concentration and retained relatively low rates, with marginal differences amongst the treatments (Table 3.4; Figure 3.5, B). The highest percentage of big shoots was obtained on media supplemented with 1.5 or 5.0 g l^{-1} glutamine – 52 and 43%, respectively and their average length was above 20 mm (Figure 3.6, A and B). The initial explants often formed roots and excretion of phenolic compounds into the media was common.

Figure 3.4: Morphology of *A. polyphylla* shoots obtained after 8 weeks in culture on media containing glutamine as a source of nitrogen at four concentrations (from left to right): 0, 0.5, 1.5 and 5.0 g l^{-1}. Bar = 10 mm.

Table 3.4: Statistical significance of the effect of glutamine concentration and NH_4^+ : NO_3^- ratio in the culture media (as independent factors) on the multiplication rate (number of shoots per explant), hyperhydricity (%) and development of shoots longer than 10 mm (%) of *A. polyphylla* after 8 weeks in culture. Glutamine concentrations (g l^{-1}): 0, 0.5, 1.5 and 5.0. NH_4^+ : NO_3^- ratios (mM): 0 : 60, 10 : 50, 20 : 40, 30 : 30, 40 : 20, 50 : 10 and 60 : 0

	F-probability level		
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)	Big shoots (%)
Glutamine concentration	< 0.001 *	0.075 ns	< 0.001 *
NH_4^+ : NO_3^- ratio	< 0.001 *	< 0.001 *	< 0.001 *

* - indicates significant treatment effect

ns – indicates non-significant treatment effect

F-probability level ≤ 0.05

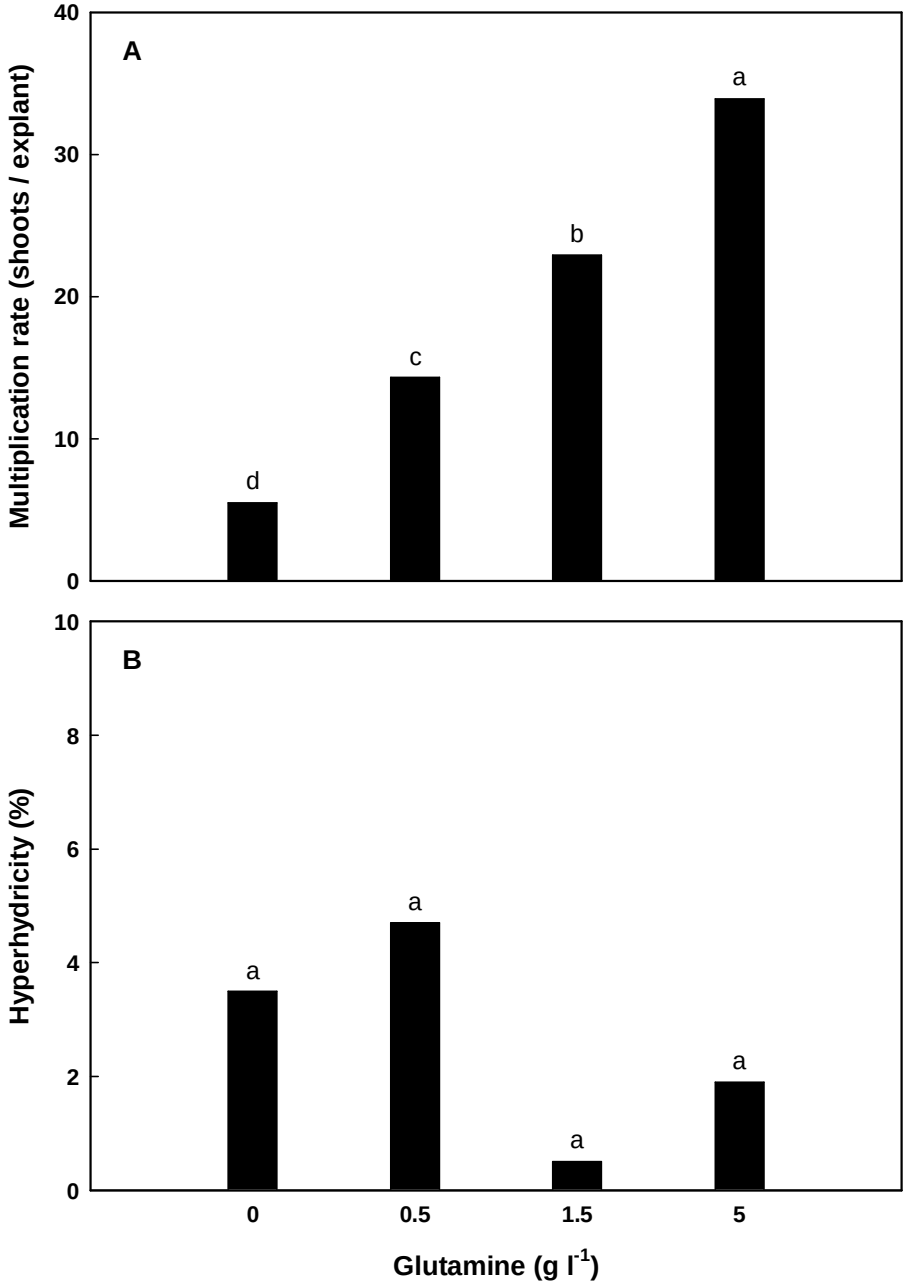

Figure 3.5: Effect of glutamine concentration in the culture media on the multiplication rate (A) and hyperhydricity (B) of *A. polyphylla* shoots after 8 weeks in culture. In (A) bars with common letters are not significantly different at $P \leq 0.05$, according to LSD test (4.6). In (B) one-way analysis of variance showed that glutamine concentration had no significant effect on hyperhydricity ($P = 0.075$).

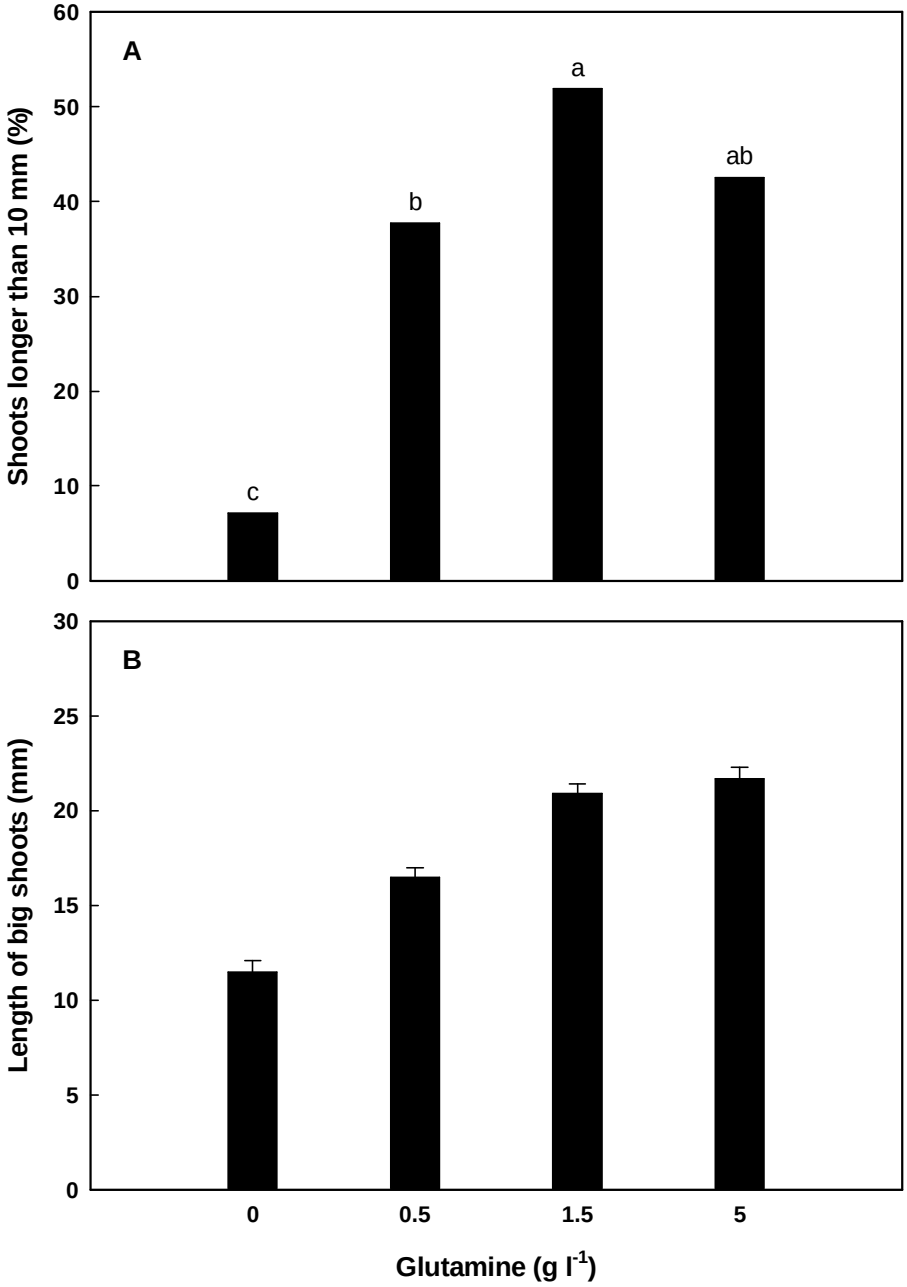

Figure 3.6: Effect of glutamine concentration in the culture media on the development of shoots longer than 10 mm (A) and the average length of shoots ≥ 10 mm (big shoots; B) of *A. polyphylla* after 8 weeks in culture. In (A) bars with common letters are not significantly different at $P \leq 0.05$, according to LSD test (7.0). In (B) means are given ± SE (n = 10 – 262).

3.3.3 Effect of ratio of NH_4^+ : NO_3^- ions

One-way analysis of variance showed that different NH_4^+ : NO_3^- ratios had a significant effect on all parameters studied: multiplication rate, hyperhydricity and production of big shoots of *A. polyphylla* after 8 weeks in culture (Table 3.4). The best shoot formation was obtained on media with a relatively equal presence of NH_4^+ and NO_3^- ions, namely ratios 20 : 40, 30 : 30 and 40 : 20 (Figure 3.8, A). The new shoots had a healthy appearance with pale green – green leaves (Figure 3.7). A further increase in the concentration of either NH_4^+ or NO_3^- ions (ratios 10 : 50 and 50 : 10) led to a significant decrease in shoot multiplication. The use of NH_4^+ or NO_3^- ions as the sole source of nitrogen (ratios 0 : 60 and 60 : 0) resulted in the lowest multiplication rate, particularly when NH_4^+ ions were employed (Figure 3.8, A). Shoots grown on media with a low ratio of NH_4^+ : NO_3^- (0 : 60 and 10 : 50) were of good quality, dark green in colour, robust and often soft white teeth were visible on the leaf margins. On the other hand, shoots from media with a high ratio of NH_4^+ : NO_3^- (60 : 0 and 50 : 10) showed poor quality, being brittle and soft, with a yellowish – pale green colour (Figure 3.7). Hyperhydricity increased progressively with the increase of NH_4^+ : NO_3^- ratio, starting at 0% (0 : 60) and reaching a maximum of 55% (60 : 0; Figure 3.8, B). Although a clear trend was not established, most of the treatments produced relatively high numbers of big shoots: 34 – 57% (Figure 3.9, A). Nevertheless, the average length of big shoots decreased gradually when raising the ratio of NH_4^+ : NO_3^- ions (Figure 3.9, B).

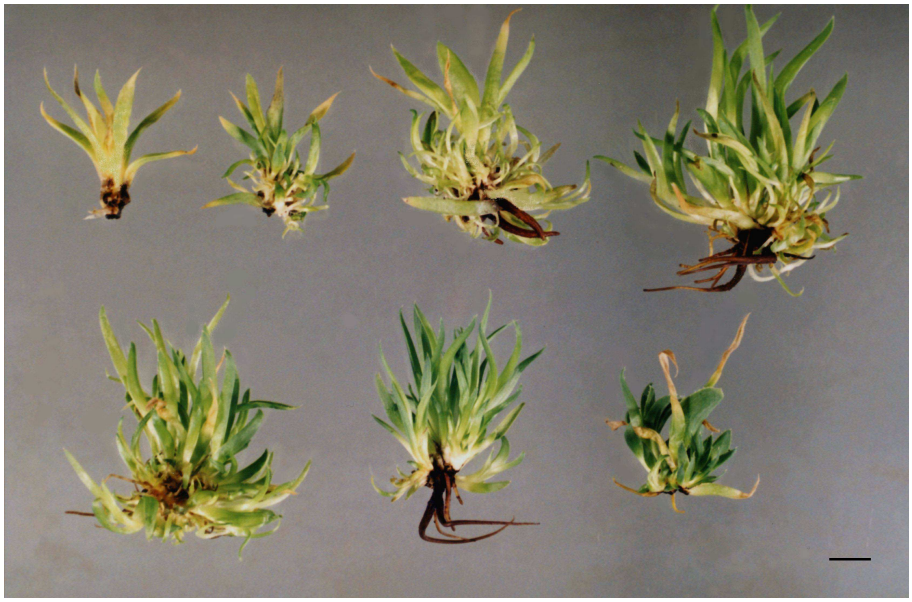

Figure 3.7: Morphology of *A. polyphylla* shoots obtained after 8 weeks in culture on media containing 60 mM total nitrogen, but a different ratio of NH_4^+ : NO_3^- (mM), namely (top row, from left to right): 60 : 0, 50 : 10, 40 : 20, 30 : 30; (bottom row, from left to right): 20 : 40, 10 : 50 and 0 : 60. Bar = 10 mm.

3.4 Discussion

A survey of the literature showed that the research on the response of cultured plant tissues to nitrogen regime in the medium has focused on several aspects of the topic:

(a) use of single defined nitrogen sources, either inorganic or organic (COUSSON and TRAN THANH VAN, 1993; AVILA *et al.*, 1994; TSAI and SAUNDERS, 1999; RAMAGE and WILLIAMS, 2002; WOODWARD *et al.*, 2006),

(b) total nitrogen provided by the inorganic ions NO_3^- and NH_4^+ and the ratio between them (FASOLO *et al.*, 1989; PREDIERI *et al.*, 1989; GRIMES and HODGES, 1990; SELBY and HARVEY, 1990; ABU-QAOUD *et al.*, 1991; LEBLAY *et al.*, 1991; ZIV and ARIEL, 1992; EVANS, 1993; NAGAKUBO *et al.*, 1993; AVILA *et al.*, 1998; LUCIANI *et al.*, 2001; LI and HAN, 2003; NOWAK *et al.*, 2007), and

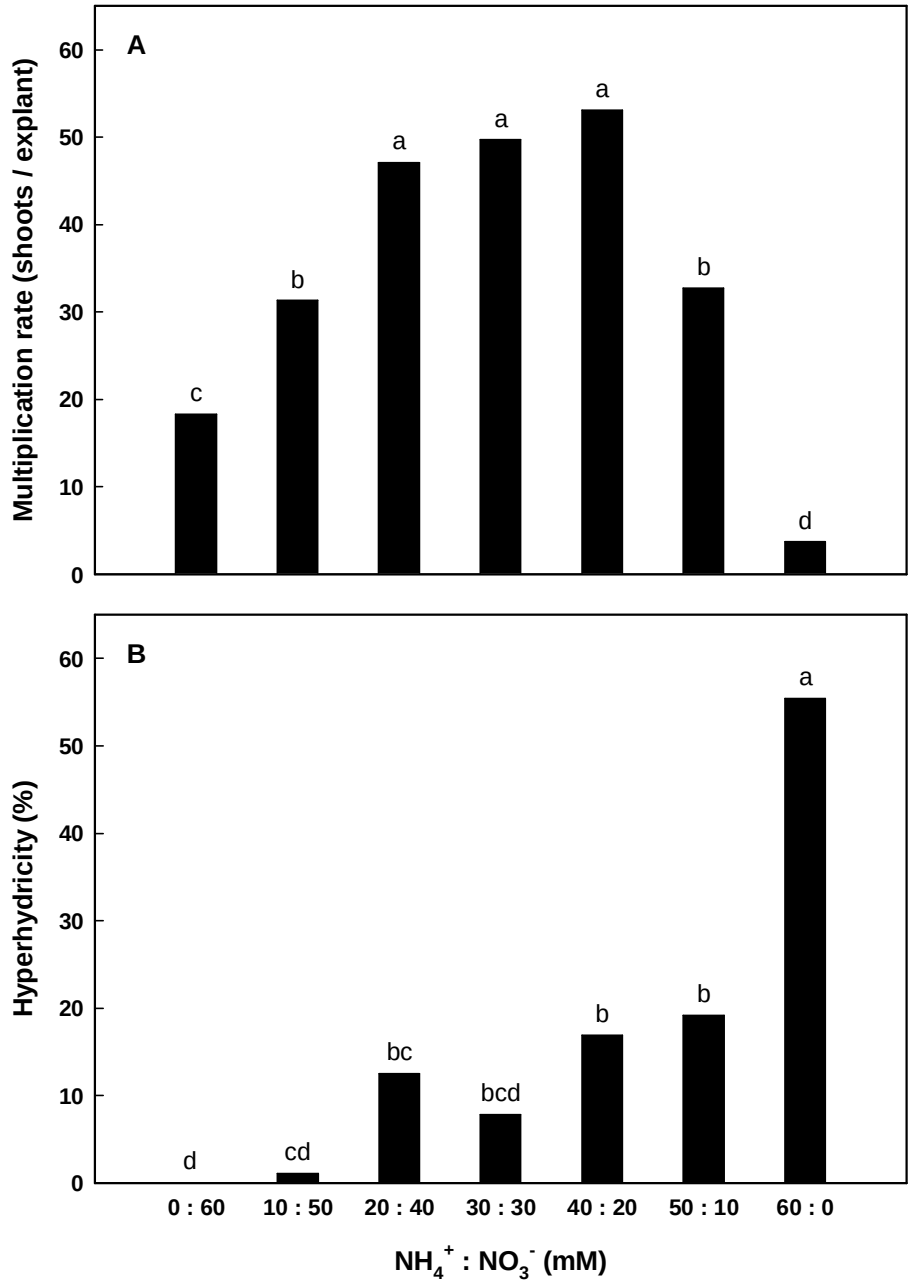

Figure 3.8: Effect of NH_4^+ : NO_3^- ratio (mM) in MS media on the multiplication rate (A) and hyperhydricity (B) of *A. polyphylla* shoots after 8 weeks in culture. Bars with common letters are not significantly different at $P \leq 0.05$, according to LSD test (6.4 – multiplication rate; 11.6 – hyperhydricity).

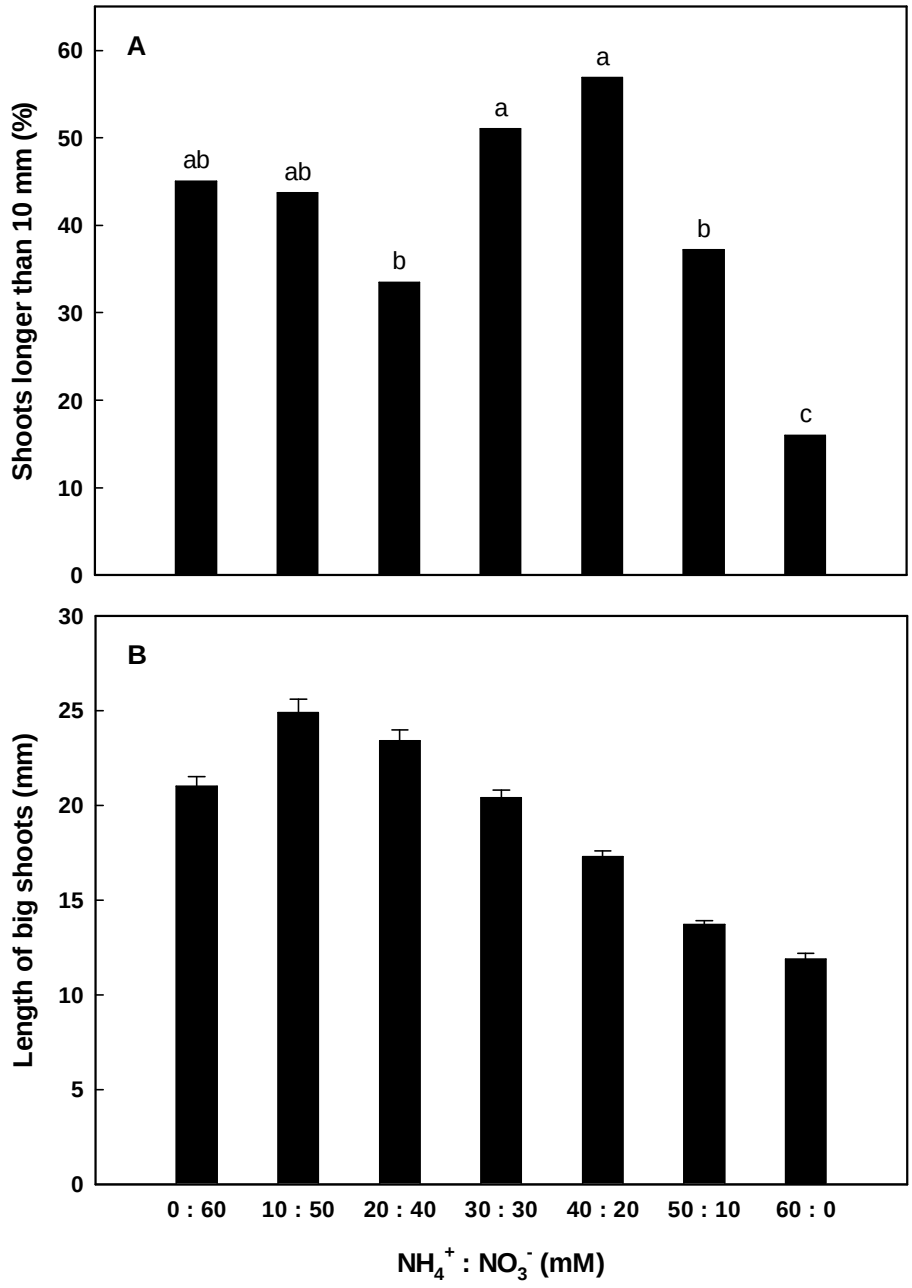

Figure 3.9: Effect of NH_4^+ : NO_3^- ratio (mM) in MS media on the development of shoots longer than 10 mm (A) and the average length of shoots ≥ 10 mm (big shoots; B) of *A. polyphylla* after 8 weeks in culture. In (A) bars with common letters are not significantly different at $P \leq 0.05$, according to LSD test (8.0). In (B) means are given ± SE (n = 15 – 674).

(c) use of organic forms of nitrogen to supplement or substitute for either NO_3^- or NH_4^+ (GAMBORG, 1970; BEHREND and MATELES, 1975; AVILA *et al.*, 1994; LELJAK-LEVANIĆ *et al.*, 2004).

The research reported in this chapter fits into the first and the second categories.

Although most plant species can utilize either form of inorganic nitrogen (NO_3^- or NH_4^+), it has been well documented that the type of nitrogen source can have a marked effect on plant growth and metabolism (GAMBORG and SHYLUK, 1970; HAYNES and GOH, 1978; BEEVERS and HAGEMAN, 1983; HAGEMAN, 1984; THORPE *et al.*, 1989).

3.4.1 Nitrate as a sole nitrogen source

Nitrate has been regarded as the principle form of nitrogen for plant tissue cultures (SATHYANARAYANA and BLAKE, 1994). In the present study nitrate as the sole source of nitrogen was sufficient for the regeneration and growth of new shoots (Figure 3.2, A). Nitrate has been shown to serve as the single nitrogen source in a number of tissue culture systems: growth and embryogenesis in carrot tissues (WETHERELL and DOUGALL, 1976), floral and vegetative organogenesis in thin cell layers of *Nicotiana tabacum* (COUSSON and TRAN THANH VAN, 1993), multiple shoot formation in garlic (NAGAKUBO *et al.*, 1993), shoot multiplication and growth in sugar beet (TSAI and SAUNDERS, 1999), meristem initiation and shoot organogenesis in tobacco leaf discs (RAMAGE and WILLIAMS, 2002), root production in banana (WU *et al.*, 2005) and *Eucalyptus marginata* (WOODWARD *et al.*, 2006). Conversely, nitrate alone did not support the regeneration of adventitious shoots from leaves of apple or pear cultivars (FASOLO *et al.*, 1989; ABU-QAOUD *et al.*, 1991) nor indica rice plant regeneration (GRIMES and HODGES, 1990). It inhibited organogenesis in *Picea sitchensis* (SELBY and HARVEY, 1990). These results clearly indicate the differential response of various plant species to the form of nitrogen supplied, reflecting the previously overlooked importance of this nutrient in determining plant morphology and morphogenesis *in vitro*.

The multiplication rate and production of big shoots in *A. polyphylla* on media with nitrate as the sole nitrogen source was not dependent on its concentration (Figures

3.2, A and 3.3, A). These results are contrary to many other studies, in which the response to nitrate was dose-dependent. For example, tobacco leaf discs cultured on 39.4 and 49.7 mM nitrate produced the greatest number of shoots, but it was considerably reduced with decreasing or increasing of the nitrate concentration (RAMAGE and WILLIAMS, 2002); callus initiation from leaf discs of sugar beet was superior on 30 mM nitrate compared with 60 mM and was inhibited on 90 mM (TSAI and SAUNDERS, 1999); increasing nitrate above 39.4 mM promoted the formation of vegetative buds instead of flowers in thin cell layers of tobacco (COUSSON and TRAN THANH VAN, 1993); the rooting of banana explants was highest in a narrow range of nitrate concentrations (17.80 to 19.78 mM) and decreased significantly outside these limits (WU *et al.*, 2005). Similarly, the highest rooting in *Eucalyptus marginata* was at 7.5 and 15.0 mM nitrate, declining markedly at 0 and 30 – 60 mM (WOODWARD *et al.*, 2006).

Information on the effect of nitrate on hyperhydricity is scarce. NAGAKUBO *et al.* (1993) reported that hyperhydricity was completely overcome on a nitrate only medium (60 mM) used for garlic shoot multiplication. This finding corroborates our data that hyperhydricity in *A. polyphylla* was entirely eliminated on media with various levels (30, 60 and 90 mM) of nitrate as the sole source of nitrogen (Figure 3.2, B).

3.4.2 Ammonium as a sole nitrogen source

Ammonium used as the sole source of nitrogen appeared to have a negative effect on regeneration and growth of new shoots of *A. polyphylla* (Figures 3.2, A and 3.3, A and B). Although nitrogen assimilation is associated with reduction of nitrate (NO_3^-) to ammonium (NH_4^+), many plant species showed inhibition of morphogenesis and growth when ammonium was supplied as the exclusive nitrogen source: *Nicotiana tabacum* (GAMBORG, 1970; COUSSON and TRAN THANH VAN, 1993; WALCH-LIU *et al.*, 2000; RAMAGE and WILLIAMS, 2002), *Oryza sativa* (GRIMES and HODGES, 1990), *Solanum tuberosum* (AVILA *et al.*, 1998) and *Eucalyptus marginata* (WOODWARD *et al.*, 2006). However, some species were found to be tolerant to sole ammonium nutrition: the increase in multiplication rate of *Beta vulgaris* shoots on medium with ammonium was equal to the increase achieved on medium supplied with nitrate only; furthermore, callus initiation from leaf discs was superior in response

to ammonium application rather than nitrate (TSAI and SAUNDERS, 1999); embryogenic cultures of *Cucurbita pepo* were initiated on medium containing ammonium as the sole source of nitrogen (LELJAK-LEVANIĆ *et al.*, 2004).

The inhibition of regeneration of *A. polyphylla* explants fed solely with ammonium was not reliant on its amount in the media (Figure 3.2, A). Similar results have been reported for *Nicotiana tabacum* and were mainly due to the fact that ammonium did not support organogenesis at any concentration it was applied at (COUSSON and TRAN THANH VAN, 1993; RAMAGE and WILLIAMS, 2002). TSAI and SAUNDERS (1999) observed a significantly higher callusing response of sugar beet leaf discs on 30 mM ammonium than on 60 mM, and WOODWARD *et al.* (2006) obtained a maximum rooting of *Eucalyptus marginata* shoots on 7.5 mM ammonium, falling considerably at higher concentrations.

The production of big *A. polyphylla* shoots and their length were significantly reduced when ammonium (NH_4^+) instead of nitrate (NO_3^-) or both ammonium and nitrate ($NH_4^+ + NO_3^-$) was supplied as the nitrogen source (Figure 3.3, A and B). These results support previous findings on potato (AVILA *et al.*, 1994; 1998), sugar beet (RAAB and TERRY, 1994) and tobacco (WALCH-LIU *et al.*, 2000). Ammonium-induced growth depression of tobacco leaves was found to be a result of both reduced cell division and cell elongation (WALCH-LIU *et al.*, 2000).

The inhibition of growth and morphogenesis in response to application of ammonium as the sole nitrogen source has been attributed mainly to (a) changes in medium pH and (b) toxic effects of free ammonium. Ammonium nutrition is associated with excess uptake of cations, which in turn is balanced by an increased net efflux of protons, resulting in acidification of the medium. A number of investigations have reported a decrease in medium pH under these circumstances (COUSSON and TRAN THANH VAN, 1993; AVILA *et al.*, 1998; RAMAGE and WILLIAMS, 2002; WOODWARD *et al.*, 2006). Low pH can lead to inhibition of root production and growth, furthermore it affects the availability of mineral nutrients in the medium, with most nutrients becoming limited when pH falls below 5 (WILLIAMS, 1993) and therefore restricting explant growth. Free ammonium ions can cause toxicity within plant tissues. To combat this effect, plant cells rapidly convert the ammonium ion into

L-glutamic acid by the amination of α-ketoglutaric acid. Therefore, ammonium supply in the medium leads to an increased amino acid and protein synthesis in the shoots, which is at the expense of carbohydrate metabolism (GEORGE *et al.*, 1987). The question arises whether the negative effects on growth and morphogenesis result from ammonium toxicity or lowering the medium pH. To answer this question for the *A. polyphylla* system further experimental conditions should be tested, particularly ammonium as the sole nitrogen source in a medium that is buffered to pH 5.8 and a control medium that is buffered to a low pH. In tobacco leaf discs the effects of pH and ammonium on meristem formation were successfully differentiated by using an organic buffer 2[*N*-morpholino]ethanesulphonic acid (MES). The frequency of meristem initiation increased from 0% on media with ammonium only to up to 88% on buffered media with ammonium as the sole nitrogen source, clearly indicating that low pH, and not ammonium, caused this inhibition (RAMAGE and WILLIAMS, 2002). Other putative growth- and morphogenesis-limiting factors in plants supplied with ammonium as the sole nitrogen provision have also been reported: (a) low availability of carbohydrates due to excessive consumption of soluble sugars for ammonium assimilation (detoxification; CRAMER and LEWIS, 1993), (b) lack of nitrate (NO_3^-) supply (WALCH-LIU *et al.*, 2000; RAMAGE and WILLIAMS, 2002) and (c) decreased biosynthesis and/or translocation of cytokinins (regulate both cell division and cell expansion) as a consequence of the absence of nitrate, which may be involved in a signal transduction pathway that regulates morphogenesis by the supply with cytokinins (WALCH-LIU *et al.*, 2000).

The frequency of hyperhydricity of ammonium-fed shoots was much higher compare to when nitrate or both ammonium and nitrate were used as a nitrogen source (Figure 3.2, B). There is no information in the literature regarding the effect of ammonium as the sole nitrogen source on hyperhydriciy. However, ammonium supplied as NH_4NO_3 at 20.6 mM has been reported to induce hyperhydricity in several studies (VIEITEZ *et al.*, 1985; DAGUIN and LETOUZÉ, 1986; ZIV *et al.*, 1987; BRAND, 1993). Raising its concentration to 61.8 mM resulted in a significant increase of hyperhydricity (IVANOVA and VAN STADEN, 2008), which is consistent with the present results. Plants grown with ammonium nutrition were found to produce abnormally high levels of ethylene compared to those grown with nitrate nitrogen (GEORGE, 1993). Excess ethylene is responsible for decreasing the activities of acid peroxidases and

phenylalanine ammonia-lyase leading to hindered lignification and reduced cell wall rigidity - symptoms typically characterising the hyperhydric state of shoots (GASPAR *et al.*, 1987). There is no established effect of medium pH on hyperhydricity. Flower buds of *Begonia* became hyperhydric when grown on a medium adjusted to pH 4.0, but the symptoms did not occur if the medium was adjusted to pH 5.0. Regardless of the initial acidity level, the pH of the medium was 4.8 – 5.0 after three weeks, indicating the immediate effect of the initial low pH on determining the hyperhydric condition (BERGHOEF and BRUINSMA, 1979). To address this putative relationship between medium pH and hyperhydricity in our experimental system, further studies should be conducted, particularly on media supplied with either ammonium as the exclusive nitrogen source or MS nitrogen mix (control media), adjusted to various initial pH levels.

3.4.3 Effect of ammonium and nitrate on regeneration and hyperhydricity of Aloe polyphylla

3.4.3.1 Effect of total concentration

In the typical regeneration medium for *A. polyphylla*, the nitrogen pool is composed of inorganic nitrate and ammonium. The present study revealed that the MS nitrogen mix was superior to any single nitrogen source for regeneration and growth of shoots (Figures 3.2, A and 3.3, A and B). One possible explanation for this is that the presence of both ions provides a partial buffering mechanism for the culture medium, leading to a more effective nitrogen uptake. The intake of these two forms of nitrogen by plants is adversely affected by the pH changes, which the ions individually induce. The preferential uptake of ammonium at high pH causes medium acidification, which in turn results in the preferential uptake of nitrate and an increase in medium pH. Several other studies also found the MS nitrogen regime more efficient than a single nitrogen source (AVILA *et al.*, 1994; TSAI and SAUNDERS, 1999; RAMAGE and WILLIAMS, 2002).

Most investigations in the literature corroborate the hypothesis that the amount of nitrogen in MS-based culture media is often too high for optimal regeneration and growth: enhanced shoot development and quality were observed on media with 30

mM or less nitrogen in chestnut (CHAUVIN and SALESSES, 1988), pear (RODRÍGUEZ *et al.*, 1991) and a few potato genotypes (EVANS, 1993; AVILA *et al.*, 1998). Conversely, in our experimental system, increasing the concentration of total nitrogen in the media led to a proportional increase in the multiplication rate (Figure 3.2, A), which was not at the expense of shoot growth and quality (Figure 3.3, A and B). Similar results were reported for *Prunus domestica* (NOWAK *et al.*, 2007).

With regard to the lower regeneration rate on media with nitrate only compared to the MS nitrogen mix (Figure 3.2, A), there are two possible explanations. Firstly, the uptake of nitrate shifts the medium pH towards alkalinity, which in turn lowers its own uptake. Secondly, nitrate reduction is energetically expensive: 15 – 16 ATP/mol compare to 5 ATP/mol for ammonium assimilation; furthermore, nitrate absorption is an active process with energetic expense (SALSAC *et al.*, 1987), whereas ammonium transport across the plasma membrane is a passive process (MIFLIN and LEA, 1976). These possibilities suggest that the presence of both ions is of mutual advantage, leading to a higher nitrogen uptake with a lower energy use, which in turn translates into a high multiplication rate.

The frequency of hyperhydricity was dose-dependant, in that the highest nitrogen concentration in the media resulted in the highest occurrence of hyperhydric shoots (HS; Figure 3.2, B). At a concentration of 60 mM (standard MS medium: 20.6 mM NH_4^+ and 39.4 mM NO_3^-), nitrogen induced hyperhydricity in *Prunus avium* (RIFFAUD and CORNU, 1981), *Castanea sativa* (VIEITEZ *et al.*, 1985), *Salix babylonica* (DAGUIN and LETOUZÉ, 1986), *Dianthus caryophyllus* (ZIV *et al.*, 1987) and *Aloe polyphylla* (IVANOVA and VAN STADEN, 2008; Chapter 2). However, the induction of hyperhydricity was attributed, by these authors, not to the nitrogen *per se*, but to the ammonium ions, which could trigger a series of events leading to this state (discussed in Chapter 2). Similar to our previous study (IVANOVA and VAN STADEN, 2008; Chapter 2), a further rise in the NH_4NO_3 considerably increased the production of HS (Figure 3.2, B). Lowering the amount of nitrogen to a half of its value in the MS medium (30 mM) eliminated hyperhydricity (BEAUCHESNE, 1981; RIFFAUD and CORNU, 1981; CHAUVIN and SALESSES, 1988). Our data corroborate these findings.

3.4.3.2 Effect of NH_4^+ : NO_3^- ratios

The specific ratio of reduced (NH_4^+) to oxidized (NO_3^-) nitrogen is an important factor for optimal plant regeneration and growth, and is furthermore species- and tissue-specific as evidenced by the plethora of media compositions for plant tissue culture. The highest multiplication rate of *A. polyphylla* was achieved on media with intermediate concentrations of ammonium and nitrate, namely ratios 20 : 40, 30 : 30 and 40 : 20, and decreased significantly outside these limits (Figure 3.8, A). The better micropropagation at these ratios may relate to minor pH fluctuations, which in turn allow access to both sources of nitrogen, rather than one or the other (see above), as well as other nutrients. Our results parallel those obtained in other studies (SELBY and HARVEY, 1990; ABU-QAOUD *et al.*, 1991; AVILA *et al.*, 1998; TSAY, 1998; RAMAGE and WILLIAMS, 2002; NOWAK *et al.*, 2007).

Lowering the NH_4^+ : NO_3^- ratio decreased considerably the frequency of hyperhydricity in the present study (Figure 3.8, B). Reduction in the occurrence of HS was achieved using the same approach in cultures of *Gypsophila paniculata* (HAN *et al.*, 1991), *Dianthus caryophyllus* (ZIV and ARIEL, 1992; TSAY, 1998) and *Allium sativum* (NAGAKUBO *et al.*, 1993). The role of ammonium in the induction of hyperhydricity via triggering a series of reactions has been established in the literature (discussed above and in Chapter 2).

3.4.4 Glutamine as a sole nitrogen source

Our results revealed that the amino acid glutamine could be used successfully as the exclusive nitrogen source for shoot production and growth of *A. polyphylla*. The response to glutamine was concentration dependent and the highest multiplication rate and growth was achieved at 5.0 g l^{-1} (Figures 3.5, A and 3.6, A and B). Glutamine was found to support the production of sugar beet embryos (SAUNDERS and TSAI, 1999) as well as callus initiation and subsequent shoot regeneration from sugar beet leaf discs (TSAI and SAUNDERS, 1999). VASUDEVAN *et al.* (2004) investigated the effect of various nitrogen sources on the multiplication in shoot tip culture of cucumber. The greatest shoot production was achieved on a medium with glutamine. One disadvantage of our experiment is that we did not use the same

concentrations for glutamine as for the other nitrogen sources, making direct comparisons between them difficult. However, 5.0 g l^{-1} glutamine, which corresponds to approximately 34 mM showed compatible results to 30 mM MS nitrogen mix (Figures 3.2 and 3.5). Nevertheless, further experiments are needed to elucidate the effect of higher glutamine concentrations on regeneration, hyperhydricity and quality of new shoots.

Almost no information on the effect of organic nitrogen sources on hyperhydricity is available. DAVIS *et al.* (1977) reported that the addition of 3 g l^{-1} casein hydrolysate to a medium containing MS salts, contributed to the hyperhydricity of carnation shoots in rotated liquid culture. Our results contradict this finding as very little hyperhydricity was observed on media with glutamine (Figure 3.5, B).

3.4.5 Nitrogen – an essential nutrient

Nitrogen, through its part in the chemistry of many organic compounds is an essential component of all living organisms. When nitrogen was omitted from the culture media shoot multiplication was very low and the growth of new shoots retarded (Figures 3.2, A and 3.3, A and B), indicating that its presence is vital for regeneration and development of *A. polyphylla* shoots. Similarly, no callus initiation and formation of meristems and shoots from leaf discs of *Beta vulgaris* and *Nicotiana tabacum* occur on nitrogen free medium (TSAI and SAUNDERS, 1999; RAMAGE and WILLIAMS, 2002). Rooting of *Eucalyptus marginata* was also insufficient upon the exclusion of nitrogen (WOODWARD *et al.*, 2006).

3.5 Conclusions and Recommendations

The present investigation showed that nitrogen is an essential nutrient for regeneration and development of *A. polyphylla* shoots. Ammonium as the sole source of nitrogen depressed shoot multiplication and growth, and escalated the frequency of hyperhydricity. Nitrate instead of ammonium as the exclusive nitrogen source resulted in eliminating hyperhydricity and increased production of shoots of fine quality. Overall, the MS nitrogen mix was superior to any single nitrogen source for regeneration and growth of shoots, suggestive of a synergistic effect between

ammonium and nitrate. However, hyperhydricity increased with raising the concentration of NH_4^+ and NO_3^- ions. Furthermore, our study revealed that not only the absolute amount of nitrogen, but also the relative amounts of ammonium and nitrate influenced the parameters tested. Best results were obtained with NH_4^+ : NO_3^- ratios (mM) of 20 : 40, 30 : 30 and 40 : 20. We demonstrated as well the potential of glutamine as a single source of organic nitrogen, since its application resulted in the production of good quality shoots and very low hyperhydricity.

Based on our results, for mass micropropagation and maintaining of stock we recommend the use of the standard MS nitrogen regime at 60 mM and NH_4^+ : NO_3^- ratios (mM) of 20 : 40, 30 : 30 or 40 : 20. Under these conditions high multiplication rates and relatively low frequency of hyperhydricity were achieved. However, for production of shoots for acclimatization, we suggest the use of 30 mM nitrate or 5.0 g l^{-1} glutamine as the sole nitrogen source. Regenerants from both treatments were of high quality: often soft white teeth developed on the leaf margins – a sign of vigorousness and root formation was normal. These features may be beneficial for their survival *ex vitro*.

Chapter 4

CONTROLLING HYPERHYDRICITY IN *IN VITRO ALOE POLYPHYLLA*: EFFECT OF GELLING AGENT, MEDIA MATRIC POTENTIAL AND CYTOKININS

4.1 Introduction

Aloe polyphylla, a member of the Asphodelaceae, is a herbaceous plant used for ornamental and medicinal purposes. Reported for being highly endangered in the wild (HILTON-TAYLOR, 1996), *in vitro* propagation has proven to be a valuable method for its conservation by producing large numbers of pathogen-free plants in a short time. However, one of the problems encountered was the occurrence of hyperhydricity, variously described as vitrification, glassiness, translucency or succulency (DEBERGH *et al.*, 1981, 1992; ZIV, 1991). Hyperhydric plants display a range of morphological, anatomical and physiological distortions, which have been described as responses to the non-wounding stress conditions found in the *in vitro* environment (KEVERS *et al.*, 1984, 2004). This phenomenon limits the multiplication and growth of plants *in vitro* and their subsequent survival rate *ex vitro*, thus resulting in significant losses to the micropropagation industry (PÂQUES, 1991; ZIV, 1991; DEBERGH *et al.*, 1992).

Understanding the role of various culture factors involved in promoting this abnormal plant development is of considerable interest. A few reports from our laboratory have already recognized some of the factors playing a role in the induction of hyperhydricity in *A. polyphylla*, as being the use of BA (CHUKWUJEKWU *et al.*, 2002), the combination of high concentrations of cytokinins and NH_4NO_3 (IVANOVA and VAN STADEN, 2008) and the high ratio of NH_4^+ : NO_3^- ions (IVANOVA and VAN STADEN, 2009). The water potential of the medium and the relative humidity of the culture atmosphere have been identified as key factors, stimulating the development of abnormal morphogenesis *in vitro* (DEBERGH, 1987). In this and the following

Chapter, we are reporting on the effects of these two aspects with special reference to overcoming hyperhydricity in *A. polyphylla*.

Gelling agents are frequently employed in plant tissue culture to impart semi-solid consistency to otherwise liquid nutrient media (SMITH and SPOMER, 1995). Agar is the most commonly used gelling agent (PUCHOOA *et al.*, 1999). It is derived from several genera of red-purple seaweeds known as agarophytes (*Gracilaria, Gelidium*; YAPHE, 1984; MARINHO-SORIANO and BOURRET, 2003). The most important drawback, however, is that agar is not a standard product (DEBERGH, 1983), showing variable quality and purity between different brands as well as batch-to-batch differences within the same brand. Furthermore, the cost of its more highly purified grades is virtually sky scraping (HUANG *et al.*, 1995). Gellan gum, Gelrite™, appears to be an economical gelling substitute. It is a product derived from bacteria (*Pseudomonas elodea*) and is characterised by a consistent quality and high purity, and substantially smaller quantities produce gels of hardness comparable to agar. The differences in the nature and features of these two types of gelling agent raised questions concerning the effect of the gelling agent *per se* on the regeneration and incidence of hyperhydricity in shoots of *A. polyphylla*.

Although gelling agents are primarily used to create semi-solidified media, they are not an “inert” medium component, but can influence the availability of water and nutrients to the cultured tissues and regulate the headspace composition (ZIV, 1991). It has been widely reported that the gelling agent type and concentration used can have a considerable effect on the performance of tissue cultured plant material (SINGHA, 1982; DEBERGH, 1983; OWENS and WOZNIAK, 1991; POCHET *et al.*, 1991; WETZSTEIN *et al.*, 1994; BERRIOS *et al.*, 1999; BERUTO *et al.*, 1999b; KLIMASZEWSKA *et al.*, 2000; PEREIRA-NETTO *et al.*, 2007; ASCENCIO-CABRAL *et al.*, 2008), including the occurrence of hyperydricity (DEBERGH *et al.*, 1981; BORNMAN and VOGELMAN, 1984; WILLIAMS and TAJI, 1991; THOMAS *et al.*, 2000; TSAY *et al.*, 2006; CASANOVA *et al.*, 2008). These physiological responses are related to the availability of water in gelled media. Water availability is affected by the matric potential of the gel, which is influenced by the gelling agent type and concentration.

There were three primary reasons for conducting the present study. The first aim was to characterise media gelled with agar or gelrite with respect to gel matric potential (a parameter, which alters media water availability). We evaluated the relation of relative matric potential to increasing gelrite concentrations and identified the concentration of gelrite that displays a relative matric potential equal to that of 8 g l^{-1} agar (the amount used in the standard multiplication medium for *A. polyphylla*). Secondly, we assessed the potential of gelrite as a possible replacement of agar with special emphasis placed on achieving high regeneration and low incidence of hyperhydricty. As the use of gelrite resulted in high hyperhydricity, our third objective was to investigate if decreasing the water availability in the media by increasing gelrite concentration was a feasible way to combat hyperhydricity. Furthermore, this evaluation was performed in combination with two other factors, which are essential for shoot organogenesis on the one hand, but could be involved in the induction of hyperhydricity on the other hand – cytokinin types and cytokinin concentrations. This approach studies not only the main effects of the factors, but estimates if the response to one factor depends on the level of another factor, thus consequently allowing the priming of abiotic factors for attaining the desired outcome: low hyperhydricity and high multiplication rates, in the case of *A. polyphylla*.

4.2 Materials and Methods

4.2.1 Plant material

In vitro grown culture of *Aloe polyphylla* was initiated from seeds in the Research Centre for Plant Growth and Development, University of KwaZulu-Natal (ABRIE and VAN STADEN, 2001). In order to multiply and maintain the culture, the resulting seedlings (shoots – in following subcultures) were subsequently cultured at an interval of eight weeks on a shoot multiplication medium. The basal medium, comprising MURASHIGE and SKOOG (1962; MS) salts and vitamins, was supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma, St. Louis, MO, USA), 5.0 µM zeatin (Sigma) and 2.46 µM IBA (Sigma). The pH of the medium was adjusted to 5.8 with 1 M KOH and 8 g l^{-1} agar (Unilab, Saarchem, South Africa) was added prior to autoclaving at 121 ℃ and 105 kPa for 20 min. The cultures were incubated under a continuous photoperiod in a growth room fitted with cool white-

fluorescent lamps (Osram L75W/20X, USA), providing a light intensity of 35 ± 2 μmol m^{-2} s^{-1}, at 25 ± 2 ℃, and a relative air humidity of appro ximately 60%. Shoots from *in vitro* grown plants were used as initial explants in the experiments described here.

4.2.2 Relative matric potential measurements

The relative matric potential of gels was measured using a modification of the method described by OWENS and WOZNIAK (1991). Gelled media were prepared using 8 g l^{-1} agar (Unilab) and a series of increasing concentrations of Gelrite (Labretoria, South Africa), namely: 1, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g l^{-1}. The basal medium consisted of MS macro- and micronutrients and the supplements used for the *in vitro* propagation of *A. polyphylla* (see above). All the media were adjusted to pH 5.8, prior to the addition of the various amounts of gelling agents, sterilized by autoclaving at 121 ℃ at 105 kPa for 20 min, distributed into 250 ml tissue culture flasks (50 ml per flask) and allowed to solidify for 24 h at room temperature. Filter paper squares (Whatman No.3; 40 mm × 40 mm) were cut and air-dried in an oven at 50 ℃ f or 24 h. After cooling down in a desiccator for 30 min, the squares were individually weighed (W_{0h}) and carefully laid on the gel surface. The flasks were closed with polypropylene screw caps and incubated at 25 ± 2 ℃ and a continuous photoperiod (as above) for 24 h, following which the filter paper was cautiously removed using forceps and weighed (W_{24h}). Each treatment was comprised of 5 replicates and the entire experiment was repeated three times. The relative matric potential was calculated using the following formula: $(W_{24h} - W_{0h}) / W_{0h}$. The values were plotted as a function of gelrite concentration and fitted with a curve described by a polynomial equation at fourth order (Figure 4.1).

4.2.3 Experimental designs

In the first experiment, two types of gelling agents were compared, namely agar and gelrite. They were applied at concentrations of 8 and 2.4 g l^{-1}, respectively, which resulted in the same relative matric potential of the media (2.09).

In the second experiment, four concentrations of gelrite (0 – liquid media, 2.4, 6 and 16 g l^{-1}) and two types of cytokinins [zeatin and BA (Sigma)], applied at three concentrations (0, 5 and 15 µM) were tested in multifactorial combinations. Altogether there were 24 treatments.

In a separate experiment, the same four concentrations of gelrite (0 – liquid media, 2.4, 6 and 16 g l^{-1}) were assessed in factorial combinations with three concentrations of the phenylurea cytokinin TDZ (Sigma; 0, 1.25 and 2.5 µM). There were 12 treatments.

4.2.4 Culture conditions

The basal MS medium was supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol and 2.46 µM IBA. The pH of all media was adjusted to 5.8 with 1 M KOH or 1 M HCl and the specific amount of gelling agent was added prior to autoclaving at 121 ℃ at 105 kPa for 20 min. The media were poured into 250 ml tissue culture flasks and allowed to solidify at room temperature. Each flask contained 50 ml of medium and was closed with a polypropylene screw cap. Explants, with an average length of 3.2 ± 0.5 cm (mean ± SD) and with five to six leaves were obtained from *in vitro* grown plantlets as specified above. Six explants were planted per flask. In the case of static liquid media, we used plastic supports with holes (d = 5 mm), which allowed only the basal tip of the explants to be immersed in the liquid media and all foliage to be kept out of liquid contact. This way the explants were prevented from submersion into the liquid media. Each treatment comprised 18 explants. All experiments were repeated at least twice. The cultures were incubated for eight weeks under the conditions described above.

4.2.5 Data collection and statistical analysis

At the end of the 8-week culture period the number of shoots per explant (multiplication rate) was recorded, and the newly-formed shoots were categorized as normal shoots (NS) or hyperhydric shoots (HS), according to their external appearance (ZIV, 1991). HS had thicker, translucent and water-logged leaves compared to NS, whose leaves showed no abnormality. Hyperhydricity (%) was

calculated based on the above information: number of HS per explant / total number of shoots per explant × 100.

The hyperhydricity (%) data were transformed into arcsine square roots prior to statistical analysis. All analyses were conducted using GenStat 9 statistical package (Release 9.1). The data on multiplication rate and hyperhydricity were analysed by t-test, two-way or multifactorial analysis of variance (ANOVA) using a completely randomised block design. Means were separated by post hoc least significant differences (LSD) test at $P \leq 0.05$.

4.3 Results

4.3.1 Matric potential measurements

The relative matric potential of gelled media was measured using air-dried filter paper as a standard matrix. Equilibrium between the paper and gel was reached within a few hours, but measurements at 24 h were chosen for convenience. In Figure 4.1 the relative matric potential is expressed as the fraction of liquid gained by the filter paper relative to the weight of the air-dry paper. Lower gelrite concentrations resulted in media with high relative matric potentials (more available water; Figure 4.1), representing potential values approaching that of pure water, traditionally given a value of 0. Media solidified with higher gelrite concentrations exhibited low relative matric potentials (less available water; Figure 4.1), representing increasingly negative potentials, indicative of stress conditions. Attempts to use gelrite concentrations lower than 1 g l^{-1} (Figure 4.1) led to artefacts, apparently caused by partial collapse of these weak gels.

Using this technique it was estimated that the relative matric potential of media solidified with 8 g l^{-1} agar (Unilab) is 2.09 ± 0.02 (mean ± SE, n = 15 replicates). At equal gel concentrations, gelrite displayed a much lower relative matric potential (Figure 4.1): 1.93 ± 0.01 (mean ± SE, n = 15) compared to agar, pointing to fundamental differences between the two gels. To determine the concentration of gelrite that displays a relative matric potential equal to that of 8 g l^{-1} Unilab agar, we

used the equation, describing the fitted curve in Figure 4.1 (R^2 = 0.9922), which is as follows:

$$y = 4E\text{-}05x^4 – 0.0018x^3 + 0.0273x^2 – 0.1806x + 2.3891.$$

As y = 2.09, the concentration of gelrite was theoretically calculated to be 2.4 g l^{-1} (see red dashed line in Figure 4.1). Using the filter paper technique, the relative matric potential of a medium prepared with 2.4 g l^{-1} gelrite was found to be 2.09 ± 0.01 (mean ± SE, n = 15), confirming the above result. Gelrite at concentration of 2.4 g l^{-1} was used in subsequent experiments.

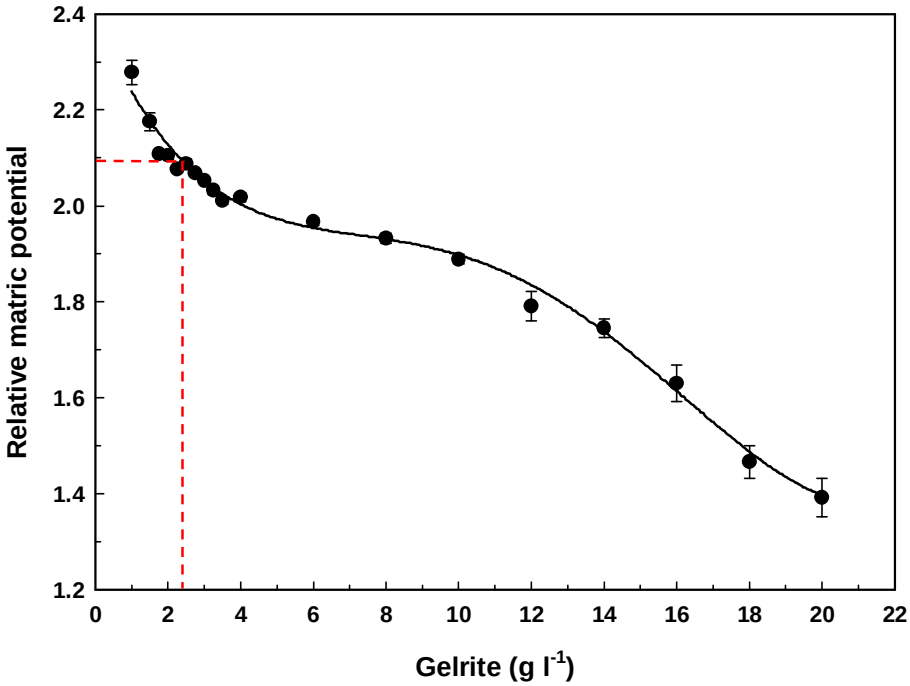

Figure 4.1: Relationship of relative matric potential to gelrite concentration. Plotted are means with SE bars. The highest relative matric potential value represents a potential value approaching that of pure free water (conventionally assigned a value of 0), and low relative matric potential values represent increasingly negative (stressed) potentials. The red dashed line indicates the concentration of gelrite that displays a relative matric potential equal to that of 8 g l^{-1} Unilab agar (2.09), namely 2.4 g l^{-1}.

4.3.2 Physiological effects of gelling agent type

In a study aiming to optimise the conditions for high regeneration rate and low occurrence of hyperhydricity of *A. polyphylla* shoots we observed large differences associated with the gelling agent employed (Table 4.1). The concentrations of agar and gelrite selected were 8 and 2.4 g l^{-1}, respectively, displaying an equal relative matric potential of the media. After 8 weeks in culture, agar resulted in a significantly higher multiplication rate and four times lower hyperhydricity compared to when gelrite was used ($P < 0.001$ for both parameters; Table 4.1). The much higher incidence of hyperhydricity on medium with gelrite (64.7%) made it the suitable gelling agent to study the effect of relative matric potential of the media (and subsequently its water availability) on hyperhydricity and shoot regeneration. These additional experiments brought further insight into explaining the major physiological differences observed with these two types of gelling agents.

Table 4.1: Effect of type of gelling agent on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *Aloe polyphylla* after 8 weeks in culture

Gelling agent [a]	Relative matric potential	Multiplication rate (shoots / explant) [b]	Hyperhydricity (%)
Agar	2.09 ± 0.02	46.8 a	17.2 b
Gelrite	2.09 ± 0.01	32.1 b	64.7 a
F-probability		< 0.001 *	< 0.001 *
LSD		6.1	14.5

[a] Agar and gelrite were applied at concentrations of 8 and 2.4 g l^{-1} respectively, resulting in the same relative matric potential of the media

[b] Means followed by common letters within a column are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test

* – indicates significant treatment effect

4.3.3 Effect of gelrite concentration and cytokinin type and concentration

Multifactorial analysis of variance showed that all factors tested, namely: gelrite concentration (and respectively the relative matric potential of the media, which was altered by the gelrite concentration; see above and Figure 4.1), cytokinin type and cytokinin concentration, and all interactions between them had significant effects on the multiplication rate of *A. polyphylla* after 8 weeks in culture (Table 4.2). Shoot regenration was observed in all combinations tested (Table 4.3). On media lacking exogenous cytokinins (0 µM) an average of 4.2 shoots per explant were produced (Table 4.3). The newly-formed shoots were of good quality with dark green leaves (Figures 4.2, A and 4.3, A). In general, lower hyperhydricity was recorded on media with no cytokinins compared to media supplemented with zeatin or BA (Figure 4.4). Almost all initial explants formed many, long and thin roots, whose production was slightly inhibited at the highest gelrite concentration (Figure 4.2, A). No or low excreation of phenolic compounds by the explants into the media was observed.

The lowest regeneration rate occurred on liquid media (0 g l^{-1} gelrite; Table 4.3). Surprisingly, the addition of cytokinins did not boost shoot organogenesis but had the opposite effect, particularly in the case of BA. The explanation for this paradox is that the presence of zeatin or BA resulted in 100 and 95%, respectively, of the initial explants becoming hyperhydric. Under this condition the majority of them lost the ability to multiply (Figures 4.2 and 4.3). Between 92 and 100% of the few newly-formed shoots were hyperhydric and brittle, with a pale green – pale brown colour (Figure 4.4, A). Hence, these combinations retained the highest incidence of hyperhydricity across all the treatments in the experiment (Figure 4.4). Root formation was rare but browning of the media was common.

Table 4.2: Statistical significance of the effect of gelrite concentration and cytokinin type and concentration on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *Aloe polyphylla* after 8 weeks in culture. Gelrite concentrations (g l^{-1}): 0, 2.4, 6 and 16; cytokinin types: zeatin and BA, applied at the following concentrations (µM): 0, 5 and 15

	F-probability level	
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)
Gelrite concentration	< 0.001 *	< 0.001 *
CK type	< 0.001 *	0.748 ns
CK concentration	< 0.001 *	< 0.001 *
Gelrite conc. × CK type	0.011 *	0.664 ns
Gelrite conc. × CK conc.	< 0.001 *	< 0.001 *
CK type × CK conc.	< 0.001 *	0.974 ns
Gelrite conc. × CK type × CK conc.	< 0.001 *	0.895 ns

* – indicates significant treatment effect
ns – indicates non-significant treatment effect
F-probability level ≤ 0.05

The best shoot formation was obtained on media solidified with 2.4 g l^{-1} gelrite and supplemented with 5 µM cytokinin (Table 4.3; Figures 4.2 and 4.3). Increasing the concentration of zeatin or BA to 15 µM inhibited considerably the multiplication rate – 1.6 and 4.2 times, respectively (Table 4.3). On media with 15 µM BA many initial explants formed black, fragile, non-organogenic callus at their bases (Figure 4.3, C). New shoots with normal appearance had a green colour, which turned into a pale green in their hyperhydric counterparts. Hyperhydricity was very high, ranging between 70 and 85%, yet slightly lower compared to liquid media (Figure 4.4, B). Almost no rooting was recorded and the media showed browning due to the release of phenolic compounds by the explants.

Table 4.3: Effect of gelrite concentration and exogenous cytokinin type and concentration on the multiplication rate (number of shoots per explant) of *Aloe polyphylla* after 8 weeks in culture

		Multiplication rate (shoots / explant) [a]	
		Cytokinin type	
Gelrite (g l^{-1})	CK conc. (µM)	Zeatin	BA
0.0	0.0	4.7 fgh	3.6 ghi
	5.0	4.9 fgh	1.2 i
	15.0	1.0 i	0.6 i
2.4	0.0	6.1 efg	5.5 fg
	5.0	30.6 a	28.7 a
	15.0	19.1 b	6.8 ef
6.0	0.0	5.1 fgh	5.0 fgh
	5.0	17.1 bc	13.6 d
	15.0	9.1 e	6.5 efg
16.0	0.0	2.1 hi	1.6 i
	5.0	14.1 cd	7.7 ef
	15.0	6.9 ef	5.1 fgh

[a] Means followed by common letters (across all the data) are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test. LSD (3.1) was calculated for the interaction between gelrite concentration, CK type and CK concentration

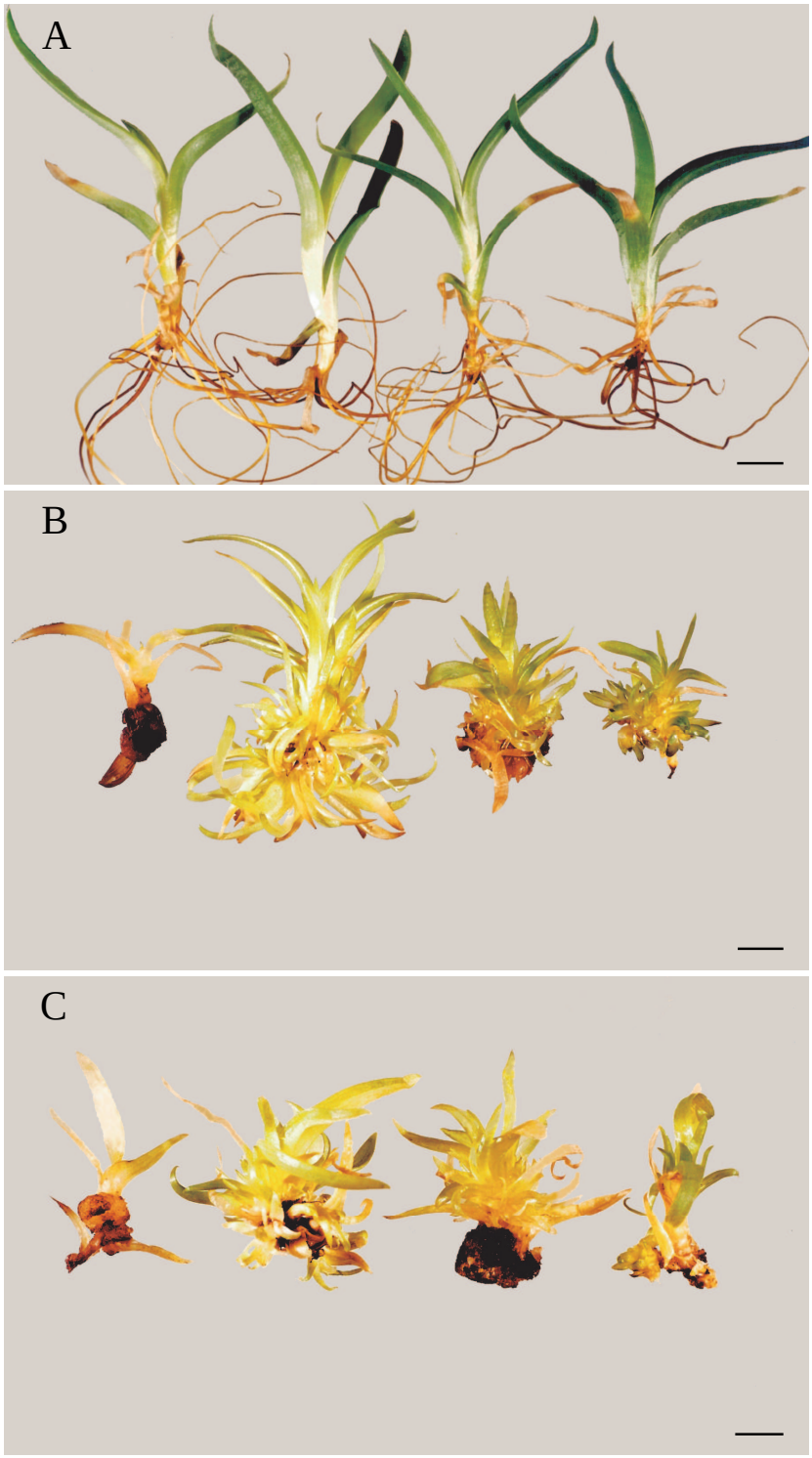

Figure 4.2: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 5 µM (B) or 15 µM (C) zeatin and solidified with gelrite at four concentrations (from left to right): 0 (liquid media), 2.4, 6 and 16 g l^{-1}. Bar = 10 mm.

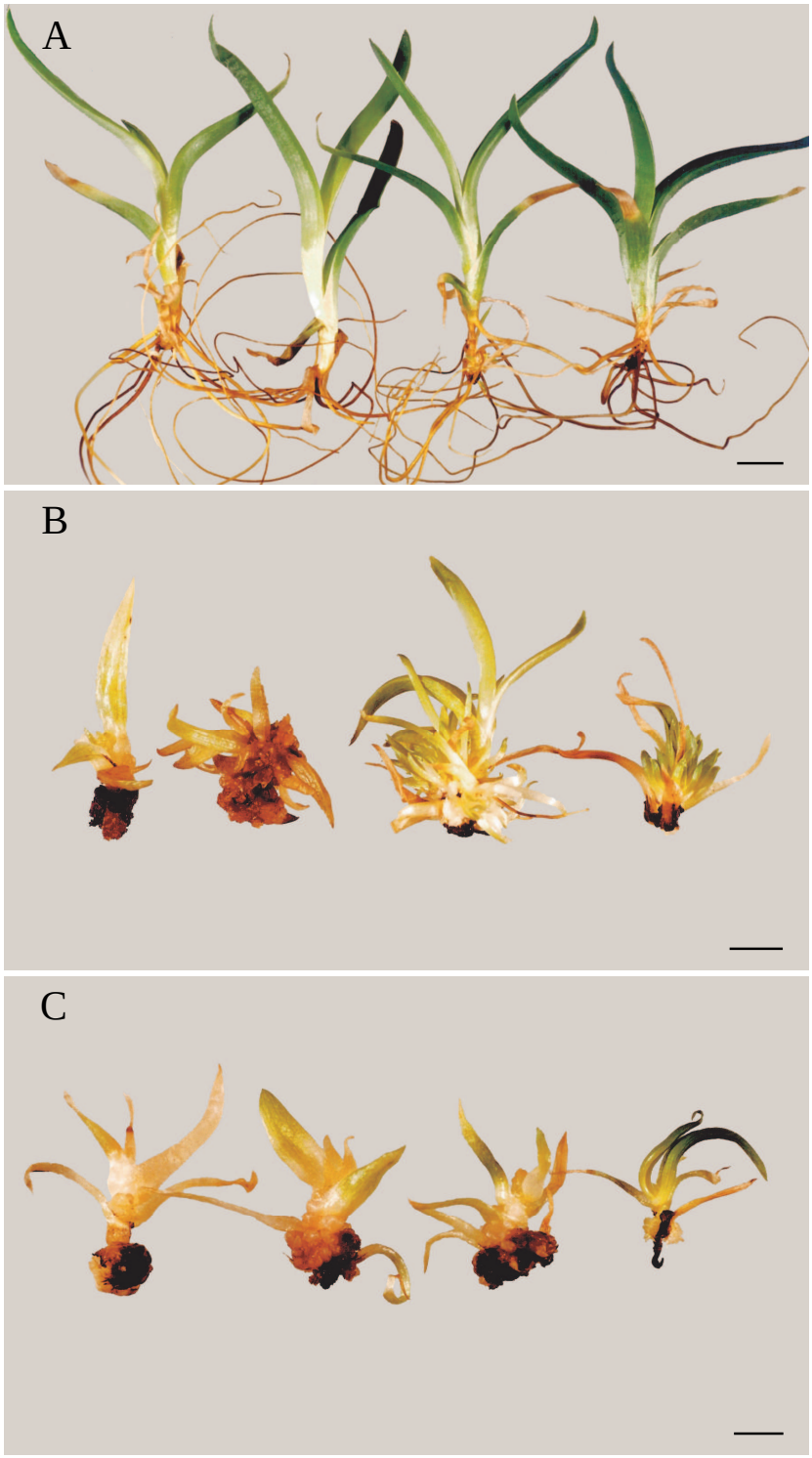

Figure 4.3: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 5 µM (B) or 15 µM (C) BA and solidified with gelrite at four concentrations (from left to right): 0 (liquid media), 2.4, 6 and 16 g l^{-1}. Bar = 10 mm.

Further increase in the concentration of gelrite to 6 and 16 g l^{-1}, which was associated with low relative matric potentials of the media and less available water respectively (Figure 4.1), resulted in a significant reduction of the multiplication (Table 4.3; Figures 4.2 and 4.3). As observed earlier, the raise in the concentration of cytokinins decreased further the regeneration of shoots (Table 4.3). Overall, in the entire experiment zeatin was shown to be the more effective cytokinin, always giving rise to a higher multiplication rate (Table 4.3). The frequency of hyperhydricity also declined progressively with the increase of gelrite concentration: 40 – 64% and 12 – 25% in the treatments with 6 and 16 g l^{-1} gelrite, correspondingly (Figure 4.4, C and D). The newly-formed shoots were green and short, particularly at the highest gelrite concentration used (length < 10 mm; Figures 4.2 and 4.3). Only few thick roots were observed as well as a faint browning of the media.

The interaction between gelrite concentration, cytikinin type and cytokinin concentration did not affect significantly the occurrence of hyperhydricity in *A. polyphylla* after 8 weeks in culture ($P = 0.895$; Table 4.2). Thus, no further separation of the means, designated by letters, was performed (Figure 4.4). However, the main effects of gelrite concentration and cytokinin concentration on hyperhydricity were significant ($P < 0.001$; Table 4.2) and are shown in Figure 4.5, A and B, respectively. Raising the concentration of gelrite correlated inversely to the frequency of hyperhydricity, which decreased gradually. On the other hand, increasing the cytokinin concentration in the media led to a proportional increase of hyperhydricity.

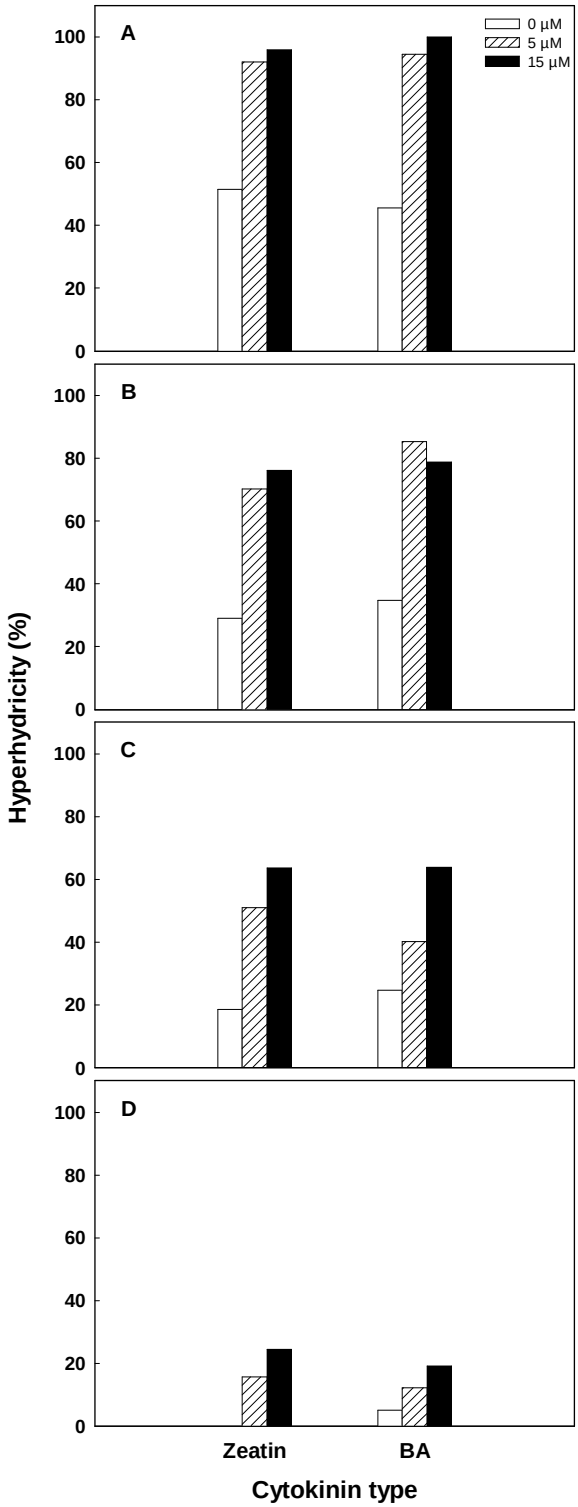

Figure 4.4: Hyperhydricity (%) of *A. polyphylla* shoots obtained after 8 weeks of culture on media solidified with 0 (liquid media; A), 2.4 (B), 6 (C) or 16 g l^{-1} (D) gelrite and supplemented with zeatin or BA, applied at 0, 5 or 15 µM. Multifactorial analysis of variance showed that the interaction between gelrite concentration, CK type and CK concentration had no significant effect on hyperhydricity (P = 0.895).

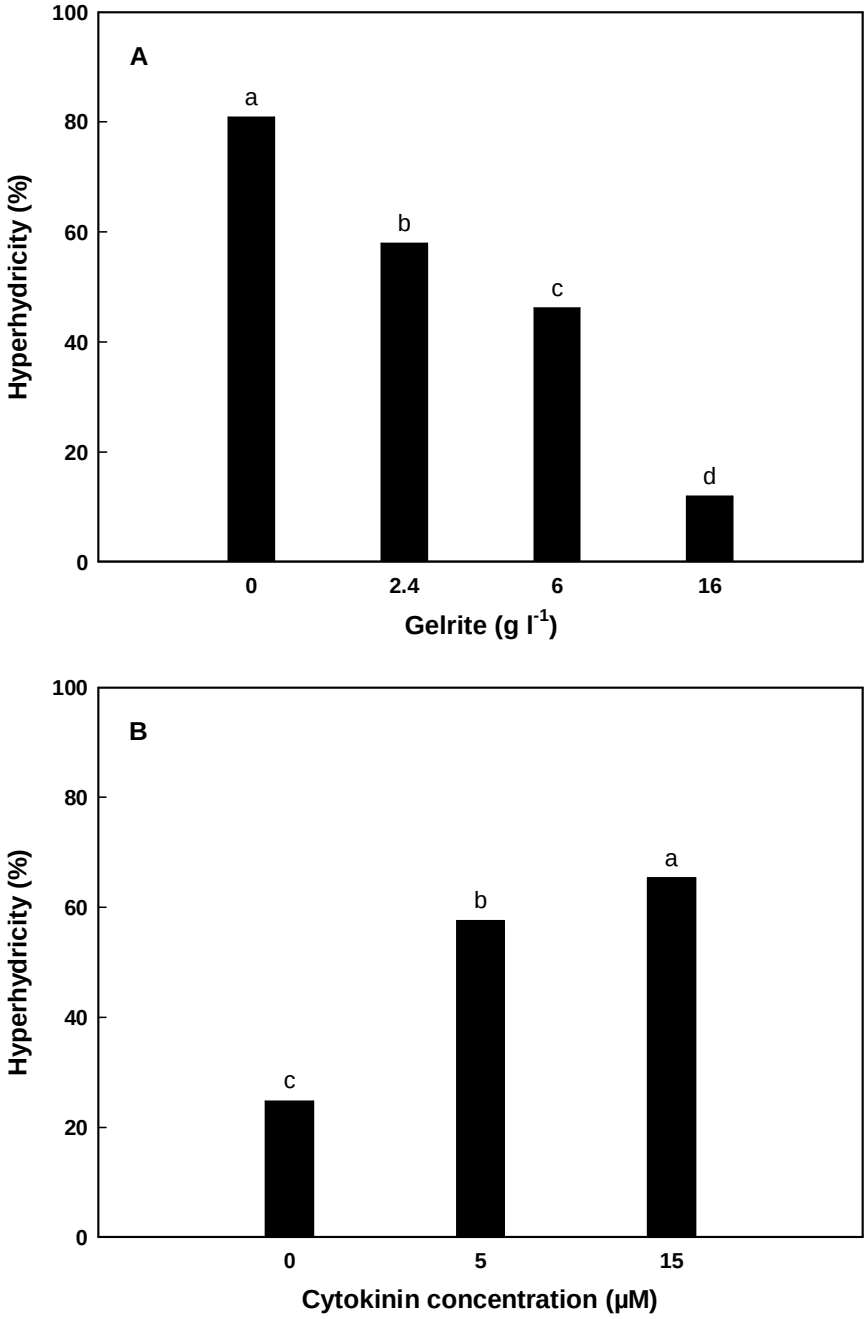

Figure 4.5: Main effect of gelrite concentration (A) and cytokinin concentration (B) on hyperhydricity (%) of *A. polyphylla* shoots after 8 weeks in culture. Bars with common letters are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test (7.3 – effect of gelrite concentration; 6.4 – effect of cytokinin concentration).

Table 4.4: Statistical significance of the effect of gelrite concentration and TDZ concentration on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *Aloe polyphylla* after 8 weeks in culture. Gelrite concentrations (g l^{-1}): 0, 2.4, 6 and 16; TDZ concentrations (µM): 0, 1.25 and 2.5

	F-probability level	
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)
Gelrite concentration	< 0.001 *	< 0.001 *
TDZ concentration	< 0.001 *	< 0.001 *
Gelrite conc. × TDZ conc.	< 0.001 *	0.081 ns

* – indicates significant treatment effect
ns – indicates non-significant treatment effect
F-probability level ≤ 0.05

4.3.4 Effect of gelrite concentration and TDZ concentration

The number of shoots per explant of *A. polyphylla* after 8 weeks in culture was significantly influenced by the interaction between gelrite concentration and TDZ concentration (Table 4.4). Overall, the multiplication rate in this experiment was very low, with 9.1 shoots per explant being the highest regeneration achieved on media solidified with 2.4 g l^{-1} gelrite and supplemented with 1.25 µM TDZ (Table 4.5). Apart from this one exception, in all other instances the addition of TDZ to the culture media led to a decrease in shoot organogenesis compared to media with no TDZ (Table 4.5). Furthermore, the frequency of hyperhydricity escalated and maintained high levels irrespectively of the gelrite concentration used (Figure 4.7). As in the previous experiment (see above), all initial explants on liquid media with TDZ became hyperhydric, loosing their ability to multiply, and even died. On media with 2.4 and 6 g l^{-1} gelrite the multiplication was slightly but significantly higher (Table 4.5). The morphological features of the plants (Figure 4.6, B and C), which are characteristic for TDZ and were also observed elsewhere (Chapter 2; IVANOVA and VAN STADEN, 2008), included: (a) swollen bases, (b) formation of callus at the base

of the initial explants, which was either sponge-like or black and fragile, (c) production of many small buds (length = 1 – 2 mm), whose growth was inhibited and they did not develop further into shoots, and (d) often the meristematic area of the plants became necrotic. Almost no rooting on media with TDZ was recorded and phenolics excretion by the explants was common.

On media with no TDZ, hyperhydricity decreased with the increase of gelrite concentration (Figure 4.7). However, in the presence of TDZ there was not a clear pattern characterising the occurrence of hyperhydricity except for being very high (Figure 4.7). Two-way analysis of variance indicated that the interaction between gelrite concentration and TDZ concentration did not have a significant effect on hyperhydricity after 8 weeks in culture ($P = 0.081$; Table 4.4) and no separation of the means was carried out (Figure 4.7). Nevertheless, the main effects of gelrite concentration and TDZ concentration on hyperhydricity were significant ($P < 0.001$; Table 4.4). Increasing the concentration of gelrite from 0 to 16 g l^{-1} resulted in a more than 50% decrease in the incidence of hyperhydricity (Figure 4.8, A). On the contrary, the addition of TDZ at any of the concentrations tested led to a 2.9-fold increase of hyperhydricity (Figure 4.8, B).

Table 4.5: Effect of gelrite concentration and TDZ concentration on the multiplication rate (number of shoots per explant) of *Aloe polyphylla* after 8 weeks in culture

	Multiplication rate (shoots / explant) [a]			
	Gelrite (g l^{-1})			
TDZ (µM)	0.0	2.4	6.0	16.0
0.00	4.3 bcd	5.8 b	5.2 bc	2.3 ef
1.25	1.0 f	9.1 a	3.7 cde	0.8 f
2.50	0.7 f	3.1 de	3.2 de	2.1 ef

[a] Means followed by common letters (across all the data) are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test. LSD (1.8) was calculated for the interaction between gelrite concentration and TDZ concentration

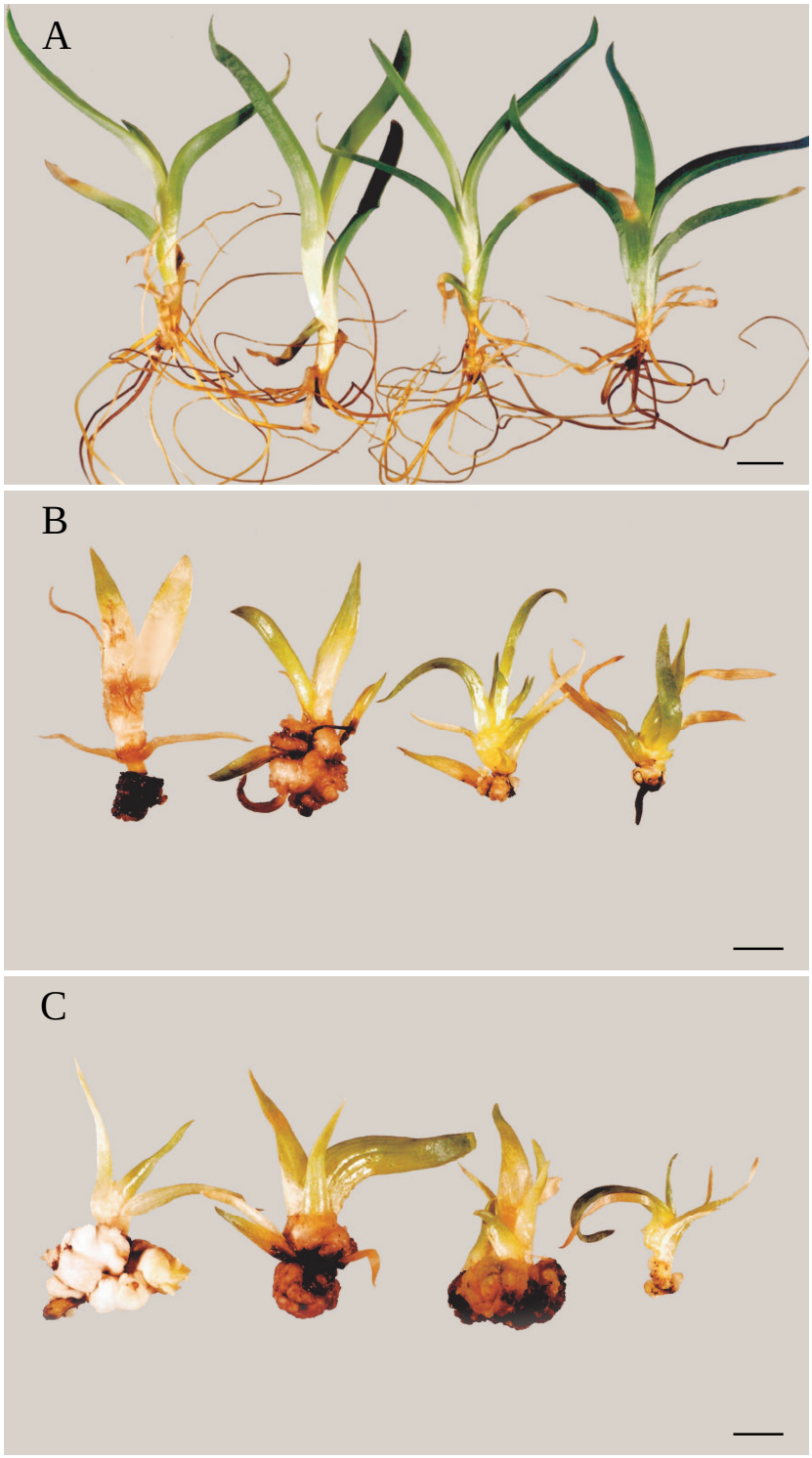

Figure 4.6: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on MS media supplemented with 0 µM (A), 1.25 µM (B) or 2.5 µM (C) TDZ and solidified with gelrite at four concentrations (from left to right): 0 (liquid media), 2.4, 6 and 16 g l^{-1}. Bar = 10 mm.

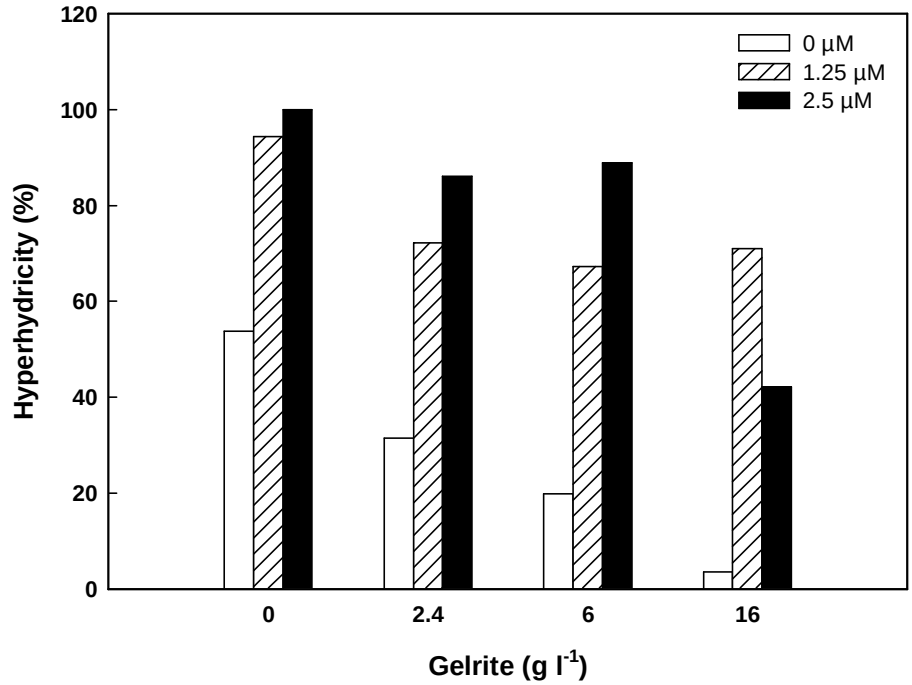

Figure 4.7: Effect of gelrite concentration and TDZ concentration on hyperhydricity (%) of *A. polyphylla* shoots after 8 weeks in culture. Two-way analysis of variance showed that the interaction between gelrite concentration and TDZ concentration had no significant effect on hyperhydricity ($P = 0.081$).

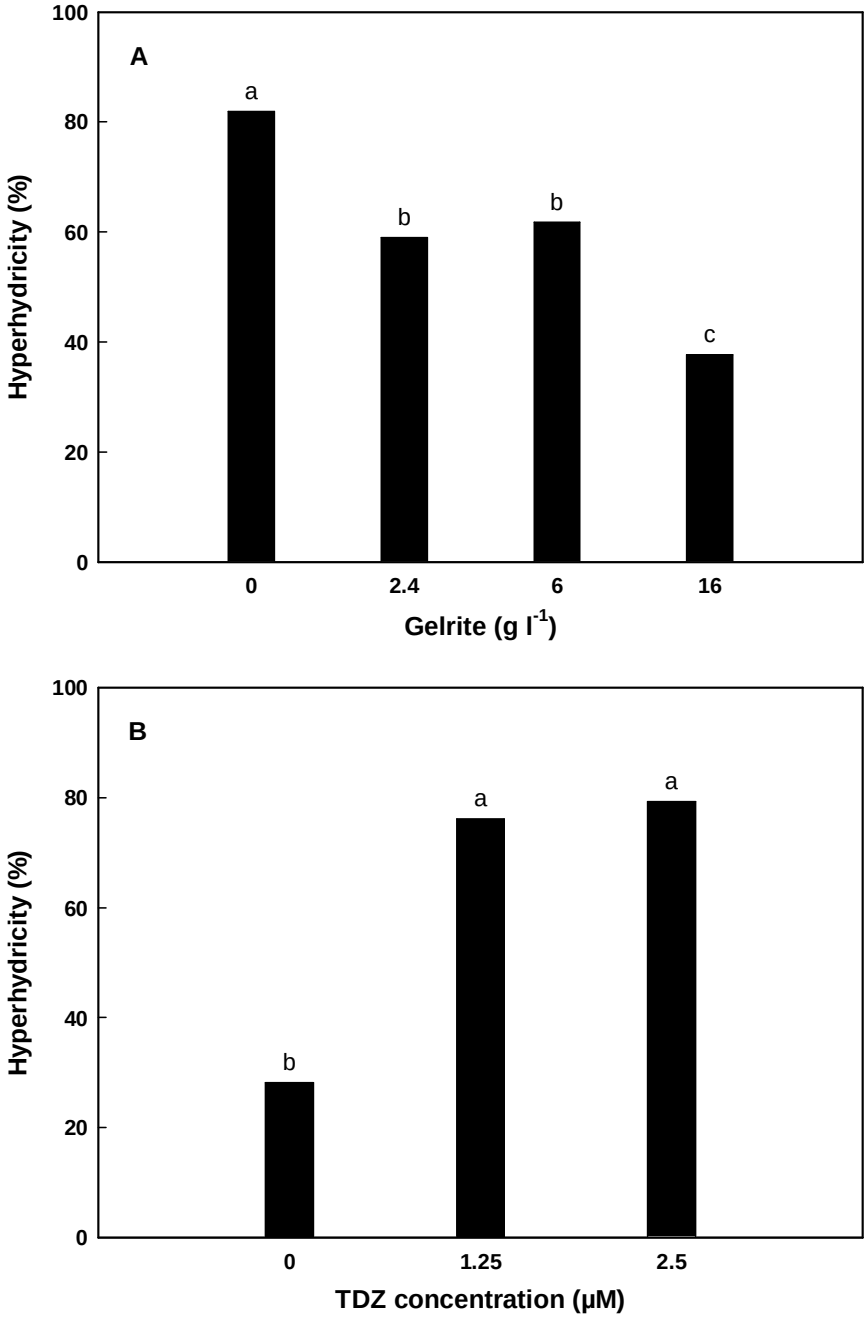

Figure 4.8: Main effect of gelrite concentration (A) and TDZ concentration (B) on hyperhydricity (%) of *A. polyphylla* shoots after 8 weeks in culture. Bars with common letters are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test (11.8 – effect of gelrite concentration; 10.2 – effect of TDZ concentration).

4.4 Discussion

4.4.1 Matric potential – a physical characteristic of gelled media

The dramatic effects of gelling agents on *in vitro* performance of cultured plant material (see Introduction for references) are closely related to the fact that gels alter water availability to the growing plant tissues (SMITH and SPOMER, 1995). Water availability can be characterised by measuring the water potential of the media. The higher the water potential of the medium, the more water is available to the explants. The major components of media water potential are osmotic (ψ_s), matric (ψ_m), gravitational (ψ_g) and pressure (ψ_p) potential (PAPENDICK and CAMPBELL, 1980). In plant tissue culture media, the matric potential component is the one that is influenced by the gelling agent type and concentration. This is the reason, for which matric potential was specifically chosen to characterise the physical state of gelled media (and indirectly the media water availability) in the present investigation.

Air-dried filter paper has long been used as a standard matrix to measure matric potentials in soil (CAMPBELL and GEE, 1986). Calibration curves have been developed to relate the water content of paper, at equilibrium with soil moisture, to water potential (McQUEEN and MILLER, 1968). From this calibration curve we estimated the gel matric potentials to be in the range of a negative few thousandths of a bar (the pressure unit, in which water potential is usually expressed), i.e. near the potential of free water, which, by convention, has 0 water potential. This result shows that the contribution of matric potential to the total water potential of gelled media is very small and corroborates previous findings (FUJIWARA, 1991; OWENS and WOZNIAK, 1991; BERUTO *et al.*, 1995). BERUTO *et al.* (1995) defined the matric potential as the maximum work required to drive the water out of the gelled medium through capillarity. Indeed, the matric potential is measured when moisture exchange between the gel and paper is through liquid phase flow. However, if the exchange is in the vapour phase, as is the case with thermocouple psychrometer measurements, then the potential measured is the sum of matric and osmotic potentials (CAMPBELL and GEE, 1986), but their respective contributions are almost impossible to differentiate (BERUTO *et al.*, 1995).

4.4.2 Media matric potential – physiological effects

The relative matric potential of the culture media decreased with increasing gelrite concentrations (Figure 4.1). Using various techniques (filter paper, tensiometer, pressure membrane, thermocouple psychrometer), several investigations have concluded that increasing the gelling agent concentration leads to a proportional decrease in the matric potential (OWENS and WOZNIAK, 1991; BHATTACHARYA *et al.*, 1994; SMITH and SPOMER, 1995; SPOMER and SMITH, 1996) or water potential of the media (CASTRO-CONCHA *et al.*, 1990; GHASHGHAIE *et al.*, 1991). The relative matric potential – gelrite concentration relationship (Figure 4.1) indicates the effect of gelrite concentration on media water availability. A reduced gel concentration will result in an increase in the water availability, but an increased gel concentration will decrease water availability to the explants (WILLIAMS, 1992; 1993).

In both of the present experiments (Figures 4.5, A and 4.8, A) decreasing the relative matric potential of the media, as represented by increasing gelrite concentrations, reduced considerably the occurrence of hyperhydricity. However, a negative side effect of this approach to overcome hyperhydricity was that it was accompanied by a decline in shoot regeneration (Tables 4.3 and 4.5). Similar results of increasing gelrite concentrations were obtained for *Picea abies* (BORNMAN and VOGELMANN, 1984), *Malus* spp. (PASQUALETTO *et al.*, 1988a; TURNER and SINGHA, 1990), *Petunia hybrida* (ZIMMERMAN and COBB, 1989), *Agave tequilana* (CASTRO-CONCHA *et al.*, 1990), *Geum quellyon* (TURNER and SINGHA, 1990), *Olearia microdisca* (WILLIAMS and TAJI, 1991) and *Pyrus pyrifolia* (KADOTA *et al.*, 2001). Furthermore, a survey of the literature indicated that the observed effects were not exclusive to the gelrite and analogous outcomes were reported when increasing concentrations of other gelling agents were used, such as agar (DEBERGH *et al.*, 1981; HAKKAART and VERSLUIJS, 1983; LESHEM, 1983a; ZIV *et al.*, 1983; BORNMAN and VOGELMANN, 1984; VON ARNOLD and ERIKSSON, 1984; BRAND, 1993; TSAY, 1998; KADOTA *et al.*, 2001; PÉREZ-TORNERO *et al.*, 2001; EBRAHIM, 2004; ABDOLI *et al.*, 2007; CASANOVA *et al.*, 2008), Gel-Gro (MONSALUD *et al.*, 1995), sago and isubgol (BHATTACHARYA *et al.*, 1994).

The effects of increasing gelling agent concentrations on hyperhydricity and regeneration ability can be ascribed to a number of reasons, some of which may act synergistically: (1) decreased media matric potential, which reduces the availability of water and mineral nutrients to the explants (discussed above; DEBERGH, 1983), (2) restricted rates of diffusion of mineral elements and macromolecules, including plant growth regulators, particularly cytokinins (DEBERGH, 1983; BORNMAN and VOGELMANN, 1984), (3) modified availability of soluble substances by means of chemical interactions with the gelling agent (BRAND, 1993), (4) increased concentrations of salts, introduced in the media as impurities in the gelling agent used (DEBERGH, 1983; BERUTO *et al.*, 1999a), and (5) constrained contact between the tissue and the culture media displaying higher strength and firmness.

Nevertheless, it has been noticed that increasing the gelrite concentration did not always result in satisfactory outcomes with respect to hyperhydricity. For example, elevating the concentration of gelrite did not overcome hyperhydricity in *Pyrus communis*, which remained 100% (TURNER and SINGHA, 1990) and in *Agave tequilana*, an acceptable frequency of hyperhydricity (18%) was achieved only when 20 g l^{-1} gelrite was used (CASTRO-CONCHA *et al.*, 1990). Similarly, the present investigation showed that raising the concentration of gelrite as high as 16 g l^{-1} could reduce sufficiently the occurrence of hyperhydricity (12%), but only on media containing zeatin or BA (Figure 4.5, A); on TDZ-supplemented media it was decreased to barely 38% (Figure 4.8, A). These rates are equal and twice as high, respectively, to the ones obtained on media solidified with 8 g l^{-1} agar (17%; Table 4.1), and exhibiting much higher relative matric potentials compared to media gelled with 16 g l^{-1} gelrite (Figure 4.1). These results suggest that overcoming hyperhydricity by increasing the concentration of gelrite is species specific and is influenced by the effect of other factors, e.g. cytokinins. Furthermore, its success is not only a result of decreased media matric potential and the consequences of it, but depends, to a large extent, on the nature of the gelling agent used.

4.4.3 Nature of the gelling agent – gelrite vs agar

Gelrite consistently produced hyperhydric shoots, even though the medium gelled with 2.4 g l^{-1} gelrite exhibited the same relative matric potential as medium with 8 g l^{-1}

agar (Unilab; Figure 4.1), on which low occurrence of hyperhydricity was observed (Table 4.1). Gelrite, as the gelling agent was found to promote and hasten hyperhydricity in *Malus domestica* (PASQUALETTO *et al.*, 1988a), *Petunia hybrida* (ZIMMERMAN *et al.*, 1991), *Swartzia madagascariensis* (BERGER and SCHAFFNER, 1995), *Allium cepa* (JAKSE *et al.*, 1996), *Dianthus caryophyllus* (TSAY, 1998) and *Pyrus pyrifolia* (KADOTA *et al.*, 2001). Substituting agar for gelrite has led to the development of a simple model system that reproducibly and easily produces hyperhydric plants within one culture cycle. This experimental system has been used to study the anatomical, morphological and biochemical differences between normal and hyperhydric shoots of apple (PASQUALETTO *et al.*, 1988b) and *Prunus avium* (FRANCK *et al.*, 1995; 1998; 2001; 2004), as well as to investigate the involvement of various culture conditions in the induction of hyperhydricity in *Olearia microdisca* (WILLIAMS and TAJI, 1991).

There are a few possible explanations for the differential performances of tissues, with respect to hyperhydricity, on media solidified with gelrite or agar. (1) The different nature and structure of the two gelling agents. Agar is derived from seaweed (agarophytes) and represents a spectrum of closely related polysaccharides belonging to the family of galactans (STANLEY, 1995). Gelrite is a bacterial polymer, composed of glucuronic acid, rhamnose, glucose and *O*-acetyl moieties (KANG *et al.*, 1982). (2) The different impurities that are introduced in the media by the gelling agent. A multielement analysis of gelrite and agar has shown quantitative and qualitative differences in their inorganic fraction (SCHERER *et al.*, 1988; WILLIAMS, 1995). (3) The different mode of gellation displayed by the two solidifying agents. Agar functions by binding water, whereas gelrite requires the presence of divalent cations in the media for gel formation. (4) The presence of components in agar that can prevent hyperhydricity. PÂQUES and BOXUS (1987b) demonstrated that hyperhydricity in liquid media can be controlled by the addition of hydrolysed agar. Fractionation of the hydrolysed agar further revealed that the most anti-hyperhydric fraction contained methylated and sulphated galactose derivatives (MARGA *et al.*, 1997). In an independent study NAIRN *et al.* (1995) identified the hydric control agent in agar as being a xylogalactan bearing pyruvate and sulphate side chains. The mechanism by which these “active constituents”, identified in both studies as sulphated polysaccharides, influence the occurrence of hyperhydricity is unclear.

Nevertheless, investigations to control hyperhydricity in both oregano (SHETTY *et al.*, 1996) and raspberry (UENO *et al.*, 1998) using an extracellular polysaccharide taken from the mucoid of a *Pseudomonas* species, appears to verify that polysaccharides are indeed performing an anti-hyperhydric role.

Few studies have reported that the concurrent use of gelrite and agar at optimal concentrations resulted in decreased hyperhydricity, good shoot regeneration and clarity and cost of the media approaching that of gelrite alone. Combinations of 1.0 – 1.5 g l^{-1} gelrite plus 2.0 – 4.0 g l^{-1} agar were found to considerably reduce the frequency of hyperhydriciy in apple (PASQUALETTO *et al.*, 1986) and onion (LUTHAR and BOHANEC, 1999), and even caused hyperhydric shoots to revert back to normal in *Petunia hybrida* (ZIMMERMAN *et al.*, 1991). Furthermore, the hyperhydricity in three apple cultivars was overcome by growing them on media solidified with a mixture of corn starch (50 g l^{-1}) plus gelrite (0.5 g l^{-1}; ZIMMERMAN *et al.*, 1995).

4.4.4 Gelled vs liquid media

When solidified, the gels form a cross-linked structure in the medium with aeration spaces. The plants grown on gelled media are provided with good physical contact with the nutrients and are also supported by the gel, and therefore prevented from submersion into a liquid medium (SMITH and SPOMER, 1995). Consequently, the use of a gelling agent helps to prevent hyperhydricity. However, this conventional technique is not preferred for scaling-up the clonal propagation as it is typically labour intensive, time consuming and expensive, and sometimes only moderately successful with regard to multiplication yields (MEHROTRA *et al.*, 2007).

On the other hand, the superior performance of cultures on liquid versus semi-solid media is well recognised (see SMITH and SPOMER, 1995 for a review). However, a major drawback is the high frequency of hyperhydricity. Despite this limitation, establishment of cultures in liquid media is requisite towards achieving automation of micropropagation schemes (AITKEN-CHRISTIE, 1991). Preventive techniques to assuage this phenomenon, yet seize the benefits of liquid media systems are therefore essential.

In the present study we investigated the potential of liquid media to sustain regeneration of *A. polyphylla*. Unfortunately, although only the basal tips of the explants were immersed in the static liquid media and all leaves were kept out of liquid contact, the occurrence of hyperhydricity was very high, particularly in the presence of cytokinins (92 – 100%; Figures 4.4, A and 4.7). The affected explants lost their ability to propagate, resulting in a very low multiplication rate (Tables 4.3 and 4.5). The high incidence of hyperhydricity in liquid media has been attributed to low oxygen content, absence of gaseous exchange and suppressed transpiration (GEORGE, 1993). Various methods have been employed to alleviate the harmful consequences of hyperhydricity in liquid media (see SMITH and SPOMER, 1995 for a review). For example, the addition of the anti-vitrification agent EM2 drastically decreased hyperhydricity in liquid cultures of strawberry (HDIDER and DESJARDINS, 1993) and *Eucalyptus* (WHITEHOUSE *et al.*, 2002).

4.4.5 Effect of cytokinins – zeatin, BA and TDZ

Both zeatin and BA were superior to TDZ for shoot regeneration (Tables 4.3 and 4.5). Increasing the concentrations of zeatin and BA, particularly, to 15 µM reduced multiplication and shoot growth (Table 4.3). Similar results have also been observed for other species (HUANG *et al.*, 1998; ARMSTRONG and JOHNSON, 2001; BOSELA and MICHLER, 2008).

The addition of cytokinins to the media aggravated the occurrence of hyperhydricity in *A. polyphylla* (Figures 4.5, B and 4.8, B). This was especially pronounced in liquid media, where between 92 and 100% of the new shoots were affected (Figures 4.4, A and 4.7). Although the amount of cytokinins was the same in liquid and semi-solid media, there would be more active cytokinin forms in the explants grown in liquid media, due to the different environmental conditions (WILLIAMS, 1992). A number of studies have shown higher endogenous cytokinin content in hyperhydric shoots (KATAEVA *et al.*, 1991; DANTAS DE OLIVEIRA *et al.*, 1997; VANDEMOORTELE *et al.*, 2001; IVANOVA *et al.*, 2006). As pointed out by HUANG *et al.* (1998), cytokinin-induced hyperhydricity may be the most difficult to overcome, since this phytohormone is usually required for shoot proliferation. The effect of cytokinins on hyperhydricity was dose-dependent (Figures 4.5, B and 4.8, B), which was consistent

with our previous findings (Chapter 2; IVANOVA and VAN STADEN, 2008). Increasing the cytokinin concentration elevated hyperhydricity in *Olearia microdisca* (WILLIAMS and TAJI, 1991), *Dianthus caryophyllus* (TSAY, 1998), *Ceratopetalum gummiferum* (ARMSTRONG and JOHNSON, 2001) and *Castanea crenata* (TETSUMURA and YAMASHITA, 2004).

In a preceding study (see Chapter 2; IVANOVA and VAN STADEN, 2008), we described a distinctive plant phenotype that was associated with the use of TDZ – abnormal leaves, swollen bases, frequent callus formation and induction of buds, which did not elongate into shoots. These morphological features were ascribed to the high levels of TDZ used (5 and 15 µM). However, similar results were obtained in the present investigation, where much lower TDZ concentrations were employed (1.25 and 2.50 µM). In addition to the low regeneration rate (Table 4.5), a high frequency of hyperhydricity was observed (Figures 4.7 and 4.8, B), making TDZ unsuitable for multiplication of *A. polyphylla*. Perhaps this is not surprising, knowing that although very effective in some herbaceous species, such as carnation (NAKANO *et al.*, 1994; CASANOVA *et al.*, 2004), this cytokinin-like substance is especially efficient with recalcitrant woody species (HUETTEMAN and PREECE, 1993; LU, 1993). TDZ-mediated hyperhydricity has also been reported as a shortcoming in several studies (HUANG *et al.*, 1998; KADOTA and NIIMI, 2003; SUNAGAWA *et al.*, 2007; BOSELA and MICHLER, 2008; DEBNATH, 2007; 2008).

4.5 Conclusions and Recommendations

The tissue culture of *A. polyphylla* in static liquid media (0 g l^{-1} gelrite) was not successful. The initial explants became hyperhydric and lost their ability to multiply. Media solidified with 2.4 g l^{-1} gelrite and supplemented with low zeatin or BA concentration (5 µM) resulted in good regeneration, however, hyperhydricity was very high. In an attempt to overcome this syndrome, decreasing media matric potentials (Ψ_m), as represented by increasing gelrite concentrations, did indeed reduce its rates but so did the multiplication. It must be noted though that tolerable incidence of hyperhydricity was achieved only when 16 g l^{-1} gelrite was used. On the other hand, the best multiplication rate and lowest hyperhydricity were observed on media with 8 g l^{-1} agar, which was equivalent to just 2.4 g l^{-1} gelrite with respect to relative matric

potential of the media. These results indicate that although there is a strong connection between matric potential and the occurrence of hyperhydricity, matric potential does not fully account for the observed effect of agar. The type of the gelling agent is therefore essential for the successful *in vitro* propagation of *A. polyphylla*. From the results presented here it is clear that gelrite is not an effective agar substitute, due particularly to the high hyperhydricity observed on media with gelrite. Furthermore, the additional research conducted here with TDZ confirmed our previous observations (see Chapter 2) that this substance with cytokinin-like activity is not suitable for the propagation of the species.

Chapter 5

HYPERHYDRICITY IN SHOOT CULTURES OF *ALOE POLYPHYLLA* CAN BE EFFECTIVELY REDUCED BY VENTILATION OF CULTURE VESSELS

5.1 Introduction

The growth and development of *in vitro* plants is affected by the composition of the nutrient medium as well as the composition of the gaseous atmosphere (KOZAI and SMITH, 1995). Explants are usually transplanted into small and closed culture vessels. Although the conventional purpose of a container closure is to maintain the aseptic conditions and prevent desiccation of the culture, it also exerts significant influence over two aspects of the *in vitro* microenvironment: the relative humidity and the gaseous composition of the headspace (SMITH and SPOMER, 1995). In closed vessels, the sufficiency of the ventilation achieved has raised major concerns (ADKINS, 1992; ARMSTRONG *et al.*, 1997). Therefore, the gaseous environment *in vitro* is often abnormal when compared to the natural *ex vitro* environment (ZOBAYED *et al.*, 1999a). The airspace of traditional tissue culture containers has been characterised with very high relative humidity, consistent temperature, low CO_2 concentration in light and high CO_2 concentration in dark, and accumulation of ethylene and other toxic substances (KOZAI *et al.*, 1992, 1996).

Plants grown *in vitro* in conventional airtight tissue culture systems often exhibit suppressed photosynthesis, transpiration and uptake of water, nutrients and CO_2, resulting in physiological and morphological disorders (DEBERGH and MAENE, 1984). The water saturated atmosphere in the culture vessels is mainly responsible for the thin cuticle development, poor epicuticular wax deposition on the leaf surface and malfunctioning stomata, leading to an absence of water loss regulation of these plants and consequently influencing their survival rate when transplanted to *ex vitro* conditions (BRAINERD and FUCHIGAMI, 1982; ZIV *et al.*, 1987). The excessive humidity *in vitro* has been suggested to be a primary reason for the hyperhydric

shoot development (ZIV, 1991). Furthermore, the abnormally high ethylene levels, exacerbated by tight vessel closures, which inhibit gas exchange, have been linked to the induction of hyperhydricity (KEVERS and GASPAR, 1985b; LEONHARDT and KANDELER, 1987; ZIV and ARIEL, 1992).

As a physiological malformation associated with *in vitro* propagation of many plant species, hyperhydricity poses a problem for the micropropagation industry due to difficulties in regeneration of normal mature plants from hyperhydric shoots (ZIV, 1991; DEBERGH *et al.*, 1992; SATO *et al.*, 1993; SHETTY *et al.*, 1995). Generally, hyperhydric leaves are light green, thick, translucent and brittle (JOHN, 1986; BÖTTCHER *et al.*, 1988). This disorder is associated with chlorophyll deficiency, poor lignification, and excessive hydration of the tissues (KEVERS *et al.*, 1984; GASPAR, 1991). In the research reported in this thesis, several approaches were used to reduce hyperhydricity in tissue culture of *Aloe polyphylla*, including media nutrient modifications (Chapters 2 and 3), hormone type and levels (Chapters 2 and 4), gelling agent type (Chapter 4), and variations in the physical properties of the culture medium (Chapter 4).

Researches have used different methods to reduce the headspace relative humidity in the culture vessels. These include increasing the gel strength of the medium (DEBERGH, 1983; SHORT *et al.*, 1987), modifying the medium osmotic potential (DAMI and HUGHES, 1996; MAKUNGA *et al.*, 2006), using medium overlays (CRANE and HUGHES, 1990), increasing the ventilation of the vessels (FAL *et al.*, 1999; MAJADA *et al.*, 2000; THOMAS *et al.*, 2000; DANTAS *et al.*, 2001; ZOBAYED *et al.*, 2002; McCARTAN *et al.*, 2004), bottom cooling the culture containers (VANDERSCHAEGHE and DEBERGH, 1987; GHASHGHAIE *et al.*, 1992; PIQUERAS *et al.*, 1998; PÉREZ-TORNERO *et al.*, 2001) and using saturated salt solutions (TANAKA *et al.*, 1992a).

From the above mentioned methods, however, only the ventilation of the culture vessels achieves both reduced relative humidity and increased gas exchange with the outer atmosphere. The easiest way to improve the *in vitro* ventilation is to change the closure of the vessels with a permeable material (BLAZKOVÁ *et al.*, 1989) or to place membranes in the culture container lids (DILLEN and BUYSENS, 1989), thus

allowing free diffusion of gases. The benefits of forced ventilation, as an alternative to diffusive ventilation, have also been extensively reported in the literature (KOZAI *et al.*, 1995; ARMSTRONG *et al.*, 1997; ZOBAYED *et al.*, 1999b, 2001), however, the systems used to achieve forced ventilation are usually rather complex.

Ventilation of tissue culture containers has three main purposes: control of hyperhydricity (HAKKAART and VERSLUIJS, 1983; DILLEN and BUYSENS, 1989; MAJADA *et al.*, 1997; LAI *et al.*, 2005), optimization of micropropagation by induction of autotrophy by CO_2 enrichment (KIRDMANEE *et al.*, 1995; KOZAI and SMITH, 1995; NGUYEN *et al.*, 1999), and *in vitro* acclimatization (ZIV, 1995; McCARTAN *et al.*, 2004; MAKUNGA *et al.*, 2006).

The aim of this part of the study was to evaluate the potential of diffusive ventilation to reduce hyperhydricity of *in vitro* regenerants of *Aloe polyphylla*. We also investigated the impact of different types of vessel closures together with gelling agent type on shoot organogenesis, growth, chlorophyll content and leaf surface ultrastructure. Our results show that ventilation of the *in vitro* container can completely eliminate hyperhydricity in *A. polyphylla*.

5.2 Materials and Methods

5.2.1 Plant material

Tissue culture of *Aloe polyphylla* was established from successfully germinated seeds by ABRIE and VAN STADEN (2001). To maintain the culture, shoots were subcultured at an interval of eight weeks on a shoot multiplication medium comprising MURASHIGE and SKOOG (1962; MS) basal medium supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma, St. Louis, MO, USA), 5.0 µM zeatin (Sigma) and 2.46 µM IBA (Sigma). The pH of the medium was adjusted to 5.8 with 1 M KOH and 8 g l^{-1} agar (Unilab, Saarchem, South Africa) was added prior to autoclaving at 121 ℃ and 105 kPa for 20 min. Six shoots were placed in tissue culture flasks (250 ml), each containing 50 ml of medium. The culture flasks were closed with polypropylene screw on lids. The cultures were incubated in a growth room at 25 ± 2 ℃, relative air humidity of approximately 60%, and under a

continuous photoperiod at a light intensity of 35 ± 2 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Osram L75W/20X, USA). Shoots from *in vitro* grown plants were used as initial explants in the experiments described here.

5.2.2 Experimental design

In the present part of the study we estimated the effect of ventilation on multiplication rate and hyperhydricity using a factorial design at four types of culture vessel closures with either agar- or gelrite-solidified media. Different ventilation was achieved by using four closures of the tissue culture containers: (i) after inoculation the culture vessels were closed with polypropylene screw on lids and sealed with two layers of Parafilm M® (American National Can, Chicago, Illinois, USA); (ii) the culture containers were closed with polypropylene screw on lids only; (iii) modified lids were used, comprising the same polypropylene screw on lids with a hole (d = 7 mm) covered with Polymon LE polyester mesh, and (iv) modified polypropylene screw on lids with a hole (d = 7 mm) covered with cotton mesh. Agar (Unilab) and Gelrite (Labretoria, South Africa) were applied at concentrations of 8 and 2.4 g l^{-1} respectively, resulting in the same relative matric potential of the media (see Chapter 4).

5.2.3 Culture conditions

The basal MS medium was supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 5.0 µM zeatin and 2.46 µM IBA. The pH of the medium was adjusted to 5.8 with 1 M KOH and solidified with either 8 g l^{-1} agar or 2.4 g l^{-1} gelrite prior to autoclaving at 121 ℃ at 105 kPa for 20 min. Fifty m l of media were poured into tissue culture flasks (250 ml) and allowed to solidify at room temperature. The culture vessels were closed using the four types of closures described above. Explants, with an average length of 3.2 ± 0.5 cm (mean ± SD) and with five to six leaves were obtained from *in vitro* grown plantlets as specified earlier. Six explants were planted per flask and each treatment comprised 18 explants. The experiment was performed twice. The cultures were incubated for eight weeks at 25 ± 2 ℃ under a continuous photoperiod (see above).

5.2.4 Data collection and statistical analysis

After eight weeks in culture the number of shoots per explant (multiplication rate) was recorded, and the newly-formed shoots were classified as normal shoots (NS) or hyperhydric shoots (HS), according to their external appearance (ZIV, 1991). HS had thicker, translucent and water-logged leaves compared to NS, whose leaves showed no abnormality. Hyperhydricity (%) was calculated based on the above information: number of HS per explant / total number of shoots per explant × 100. The average length of new shoots (mm) for individual treatments was estimated as well.

The hyperhydricity (%) data were transformed into arcsine square roots prior to statistical analysis. The data on multiplication rate and hyperhydricity were subjected to two-way analysis of variance (ANOVA) using a completely randomised block design. Significant treatment means were separated by least significant difference (LSD) test at a 5% probability. All analyses were performed using GenStat 9 statistical software (Release 9.1).

5.2.5 Chlorophyll determination

Chlorophyll was extracted in *N, N*-dimethylformamide as described by MORAN and PORATH (1980). Intact leaf samples were directly immersed in the solvent and left for 48h at 4 ℃ in the dark. The ratio for the extra ction was 5% (w/v). The absorbance of the extracts was read at λ = 647 nm and λ = 664.5 nm using a Cary 50 Conc UV-Visible spectrophotometer (Varian Pty Ltd, Victoria, Australia). Chlorophyll content calculations were based on the extinction coefficients proposed by INSKEEP and BLOOM (1985): Chl = 17.90 × A_{647} + 8.08 × $A_{664.5}$, where Chl – chlorophyll in mg l^{-1} and A – absorbance in 1 cm quartz cuvette at the specified wavelength (nm). The results represent the mean values of two different experiments with six replicates each.

5.2.6 Ultrastructural analysis: environmental scanning electron microscopy

Leaf samples of *A. polyphylla* shoots from various treatments were obtained after 8 weeks in culture. Samples from both normal shoots and hyperhydric shoots within

individual treatments were collected wherever possible. Four leaf samples (*ca.* 3 mm × 3 mm) were taken from the upper half of fully expanded external leaves. Both adaxial and abaxial leaf surfaces were examined and micrographed using a Philips XL30 environmental scanning electron microscope (ESEM) at 15 kV.

5.2.7 Characteristics of culture media

Relative water loss (RWL) from the media was calculated for the four different types of culture vessel closures by gravimetric methods. The following formula was used:

$$\text{RWL (\%)} = \frac{W_{0d} - W_{xd}}{W_{0d} - W_{tcf}} \times 100$$

where W_{0d} – weight of tissue culture flask and medium at the start of the experiment (day 0); W_{xd} – weight of tissue culture flask and medium at day *x*; and W_{tcf} – weight of tissue culture flask.

We also determined the increase of agar concentration (IAC) as well as the increase of gelrite concentration (IGC) according to DILLEN and BUYSENS (1989):

$$\text{IAC (\%) or IGC (\%)} = \left(\left(\frac{100}{(100 - \text{RWL})} \right) - 1 \right) \times 100.$$

IAC and IGC were converted from % into g l^{-1} by taking into consideration the initial concentration of agar and gelrite used to solidify the media.

RWL, IAC and IGC were estimated at regular intervals during the 8-week culture period. The culture containers were kept under the same growth room conditions as the *in vitro* culture (see above). Five replicates were used per treatment and the experiment was repeated twice. The mean values were plotted as a function of time and fitted with linear or exponential curves (Figures 5.7 and 5.8).

5.3 Results

5.3.1 Effect of ventilation and gelling agent on regeneration and hyperhydricity

The four types of culture container closures, providing different degrees of ventilation, and the type of gelling agent, as well as the interaction between these two factors influenced significantly shoot regeneration and hyperhydricity of *A. polyphylla* after 8 weeks in culture (Table 5.1). The highest multiplication rate was recorded on media with agar in non-ventilated culture vessels closed with sealed lids and lids only, averaging at 39.5 shoots per explant (Figure 5.1, A). The occurrence of hyperhydricity was relatively low (Figure 5.1, B). Substituting agar with gelrite reduced the propagation, but it still remained fairly high (Figure 5.1, A). Hyperhydricity escalated under these conditions and affected more than 70% of the newly-formed shoots (Figures 5.1, B and 5.2, B). The use of modified lids with a hole covered with polyester or cotton mesh and allowing ventilation of the culture containers resulted in a 2- to 3-fold decrease in shoot regeneration and complete elimination of hyperhydricity (Figures 5.1 and 5.2). During the second half of the culture period in ventilated culture vessels with gelrite, a spontaneous reversion of semi-hyperhydric shoots to normal was observed. The glassy appearance was not any longer evident at the end of the culture period and the watery callus that formed at the base of the explants (a typical feature of hyperhydric explants) had dried and often roots were formed (Figure 5.2, B).

Table 5.1: Statistical significance of the effect of gelling agent type and closure of the tissue culture container on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *Aloe polyphylla* after 8 weeks in culture. Type of gelling agent: agar and gelrite; closure of the culture vessel: (i) polypropylene screw on lid, sealed with Parafilm, (ii) polypropylene screw on lid only, (iii) modified polypropylene screw on lid with a hole (d = 7 mm), covered with polyester mesh, and (iv) modified polypropylene screw on lid with a hole (d = 7 mm), covered with cotton mesh

	F-probability level	
Treatment	Multiplication rate (shoots / explant)	Hyperhydricity (%)
Gelling agent	< 0.001 *	< 0.001 *
Culture container closure	< 0.001 *	< 0.001 *
Gelling agent × Container closure	< 0.001 *	< 0.001 *

* – indicates significant treatment effect

F-probability level ≤ 0.05

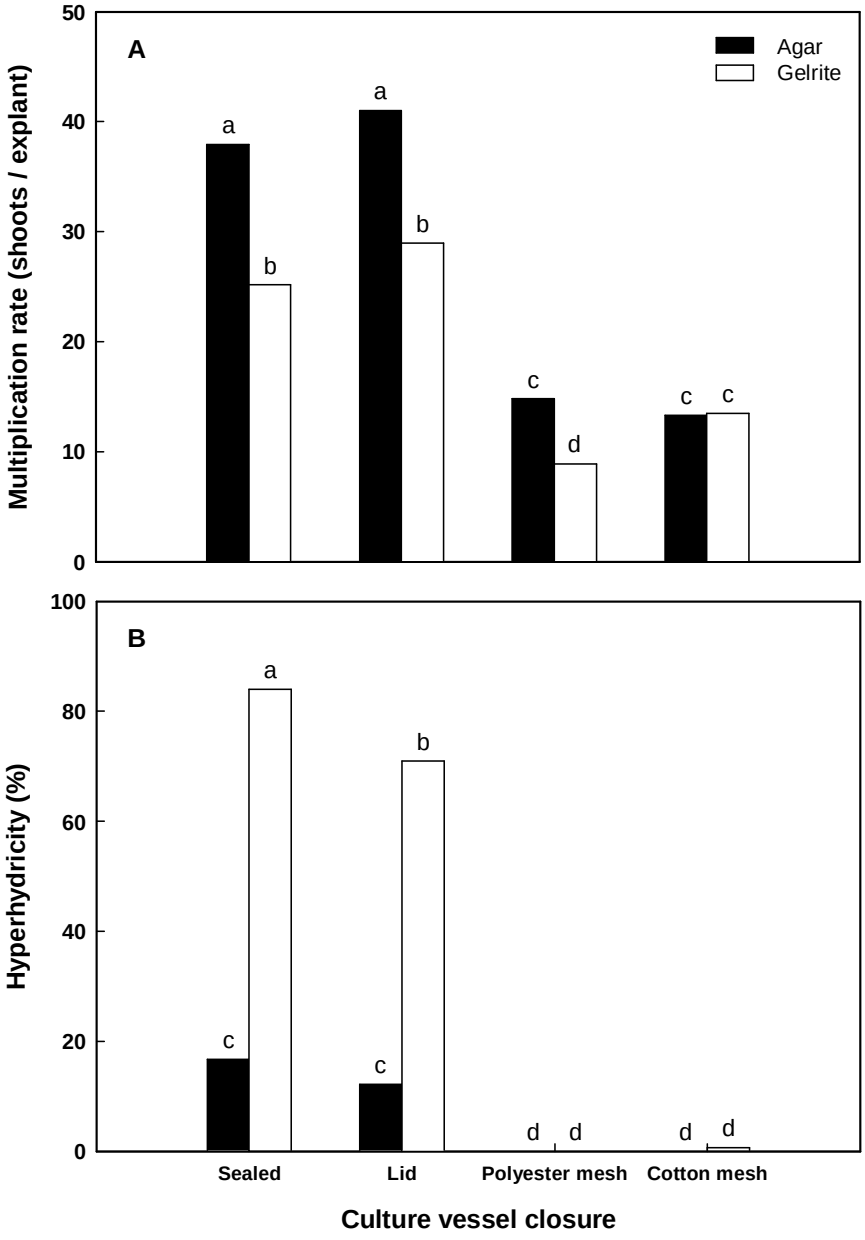

Figure 5.1: Effect of gelling agent and culture vessel closure on the multiplication rate (A) and hyperhydricity (B) of *A. polyphylla* shoots after 8 weeks in culture. The different closures of the tissue culture containers are denoted as: 'Sealed' – lid sealed with Parafilm; 'Lid' – lid only; 'Polyester mesh' – modified lid with a hole (d = 7 mm), covered with polyester mesh; and 'Cotton mesh' – modified lid with a hole (d = 7 mm), covered with cotton mesh. Bars with common letters are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test. LSD (3.9 – multiplication rate; 6.8 – hyperhydricity) was calculated for the interaction between gelling agent and culture container closure.

Figure 5.2: Morphology of *A. polyphylla* shoots obtained after 8 weeks of culture on media solidified with agar (A) or gelrite (B), in tissue culture containers with different closures (from left to right): polypropylene screw on lid, sealed with Parafilm; polypropylene screw on lid only; modified polypropylene screw on lid with a hole (d = 7 mm), covered with polyester mesh; and modified polypropylene screw on lid with a hole (d = 7 mm), covered with cotton mesh. Bar = 10 mm.

5.3.2 Effect of ventilation and gelling agent on shoot length and chlorophyll content

The main pattern observed was that shoot length decreased with increasing ventilation of culture vessels (Figures 5.2 and 5.3). However, when gelrite was used the differences were very small (Figure 5.3). This could be due to the following reasons: (i) between 71 and 84% of shoots from non-ventilated containers were hyperhydric (Figure 5.1, B) and their growth was hindered, and (ii) the ultrastructural analysis of ventilated shoots revealed much elongated epidermal cells. The longest shoots, obtained from sealed containers, had spindly and frail leaves with a bright green colour (Figures 5.2 and 5.3). In contrast, shoots from ventilated cultures were robust and dark green, particularly the ones grown on agar, where the shortest shoots were recorded as well (Figures 5.2 and 5.3).

The results on chlorophyll content of shoots from various treatments showed two apparent trends. Firstly, shoots grown on agar had a 2- to 3-times higher chlorophyll content than plants from medium with gelrite under the same ventilation conditions (Figure 5.4, A). Secondly, increasing culture ventilation increased substantially the chlorophyll levels (Figures 5.4, A). These patterns were also evident from Figure 5.2, which shows the general morphology of the shoots. The lowest chlorophyll content was measured in hyperhydric shoots grown on media with gelrite in vessels closed with sealed lid or lid only (Figures 5.4, A and 5.2). Normal shoots from medium with agar and culture vessels caped with modified lids with a hole covered with polyester mesh showed the highest chlorophyll values (Figures 5.4, A and 5.2). Within an individual treatment, hyperhydric shoots displayed 2.5-times lower chlorophyll levels compared to their normal counterparts (Figure 5.4, B).

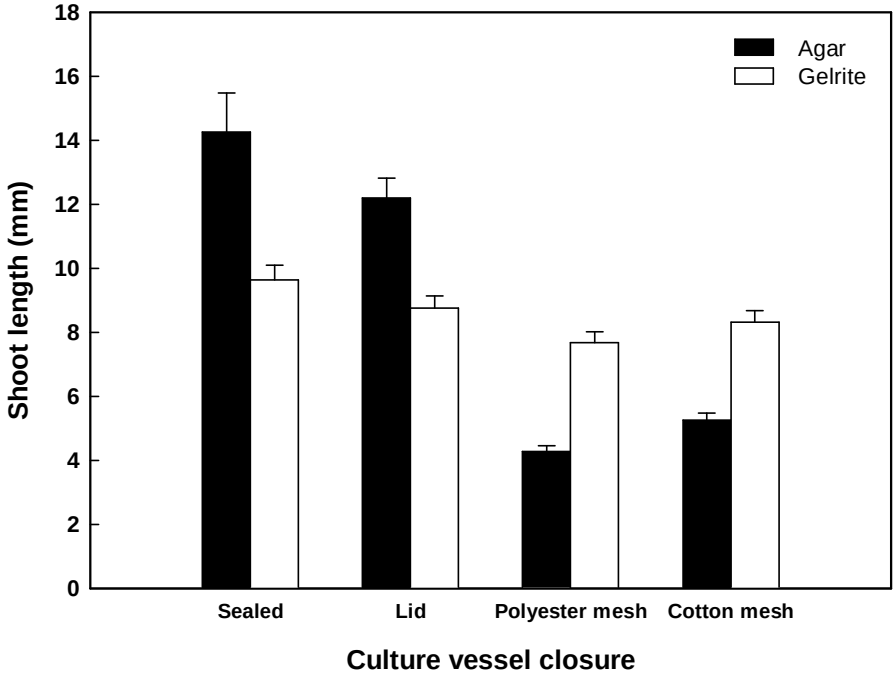

Figure 5.3: Shoot length (mm) of *A. polyphylla* shoots obtained after 8 weeks in culture on media with agar or gelrite, in culture vessels with different closures denoted as: 'Sealed' – polypropylene screw on lid sealed with Parafilm; 'Lid' – polypropylene screw on lid only; 'Polyester mesh' – modified polypropylene screw on lid with a hole (d = 7 mm), covered with polyester mesh; and 'Cotton mesh' – modified polypropylene screw on lid with a hole (d = 7 mm), covered with cotton mesh. Bars represent means ± SE (*n* = 140 – 392).

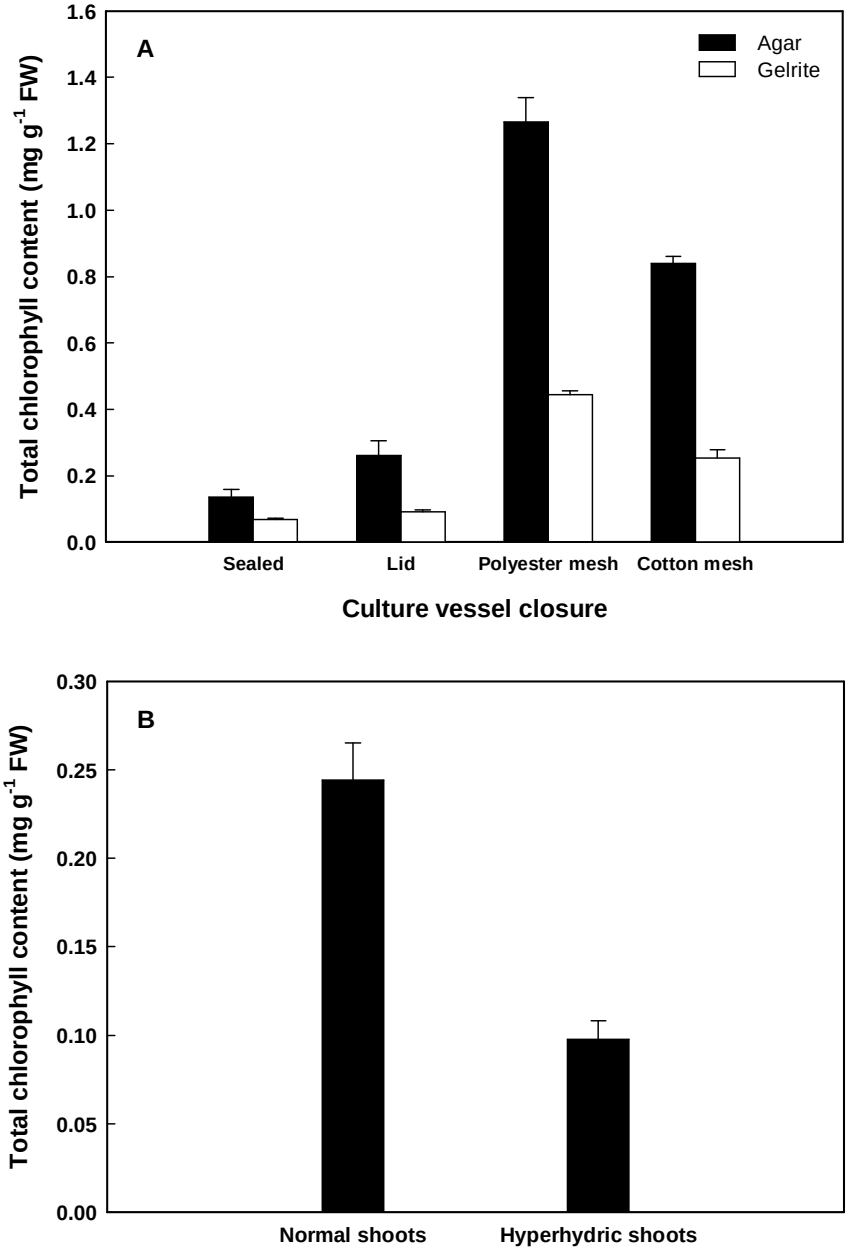

Figure 5.4: Total chlorophyll content of *A. polyphylla* shoots after 8 weeks in culture. (A) Shoots grown on media with agar or gelrite, in culture vessels with different closures denoted as: 'Sealed' – lid sealed with Parafilm; 'Lid' – lid only; 'Polyester mesh' – modified lid with a hole (d = 7 mm), covered with polyester mesh; and 'Cotton mesh' – modified lid with a hole (d = 7 mm), covered with cotton mesh. (B) Normal shoots and hyperhydric shoots obtained from medium solidified with agar and a culture container closed with lid only. Bars represent means ± SE (*n* = 12).

5.3.3 Ultrastructural analysis

In shoots of *A. polyphylla*, obtained from treatments allowing the formation of surface wax, epicuticular waxes were deposited on the adaxial and abaxial leaf surfaces (Figures 5.5, B and 5.6, D and F). Similarly, stomata always occurred on both leaf surfaces, situated slightly lower than the level of ordinary epidermal cells (Figures 5.5 and 5.6). Normal shoots and hyperhydric shoots grown on medium with agar in culture vessels closed with lids only (standard conditions for multiplying and maintaining the culture; see above), were compared (Figure 5.5). The epidermis of normal shoots displayed a rough and uneven texture, while the hyperhydric surface was very smooth (Figure 5.5, A and C). Two types of epicuticular waxes were observed in normal shoots – needle-shaped and crystalline, however, they were absent on hyperhydric leaf tissues (Figure 5.5, B and D). The frequency of the stomata was higher in normal shoots and they displayed normal morphology. Hyperhydric leaves had larger stomata, which were open, reflecting possible alterations of the overall stomata functioning (Figure 5.5).

Increasing the ventilation of culture containers by using different type of closures, namely lids sealed with Parafilm, lids only and modified lids with a hole covered with polyester mesh, resulted in various changes of the leaf surface (Figure 5.6): (i) the texture of the epidermal layer transformed from level and smooth in leaves from sealed vessels, to uneven, and finally to rough and bumpy in the most ventilated shoots (Figure 5.6, A, C and E); (ii) the frequency of stomata increased; (iii) the size of stomata decreased with increasing ventilation; (iv) stomata displayed different morphology: open – in non-ventilated environment, and closed and sunken – in ventilated containers (Figure 5.6, F); (v) wax deposition improved with better ventilation – while it was absent in shoots from sealed vessels, it appeared scattered in leaves with limited ventilation and dense in well ventilated plants; and (vi) the degree of ventilation also affected the type of epicuticular wax formed – needle-shaped and crystalline waxes were typical for shoots from culture containers closed with lids (Figure 5.6, D), but only elongated needle-like waxes were observed in shoots grown under high ventilation (Figure 5.6, F).

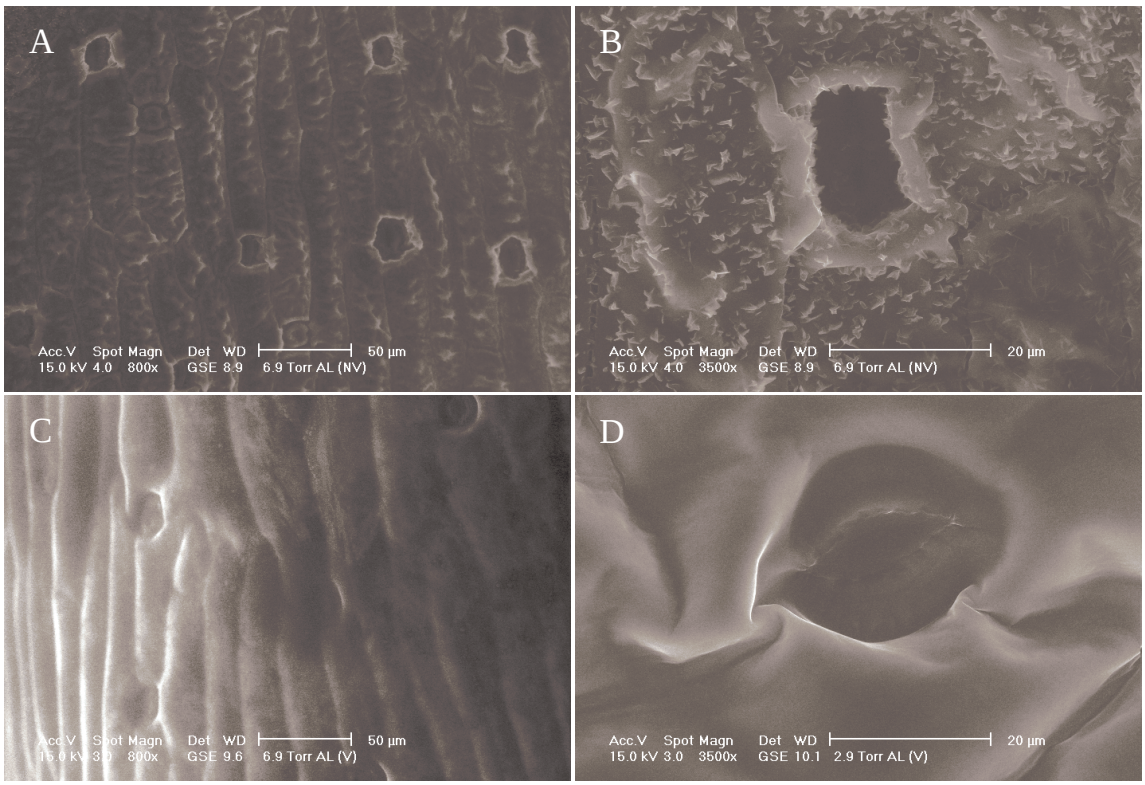

Figure 5.5: Environmental scanning electron microscopy of leaves of normal shoots (A and B) and hyperhydric shoots (C and D) of *A. polyphylla* grown for 8 weeks on medium with agar, in a culture container closed with a polypropylene screw on lid only. View of the adaxial leaf surface showing the epidermis, numerous stomata and the presence (B) or absence (D) of epicuticular wax.

5.3.4 Effect of ventilation and gelling agent on the physical characteristics of culture media

The water loss from the culture media depended on both the type of vessel closure and the type of gelling agent. High relative water loss was observed from media in flasks closed with modified lids with a hole and there was no significant differences if the hole in the lid was covered with polyester mesh or cotton mesh (Figure 5.7). However, the relative water loss from ventilated vessels was higher in medium with gelrite (49.3 – 53%) than in medium solidified with agar (42 – 44%; Figure 5.7, A and B). Very little water loss was recorded from non-ventilated cultures (Figure 5.7).

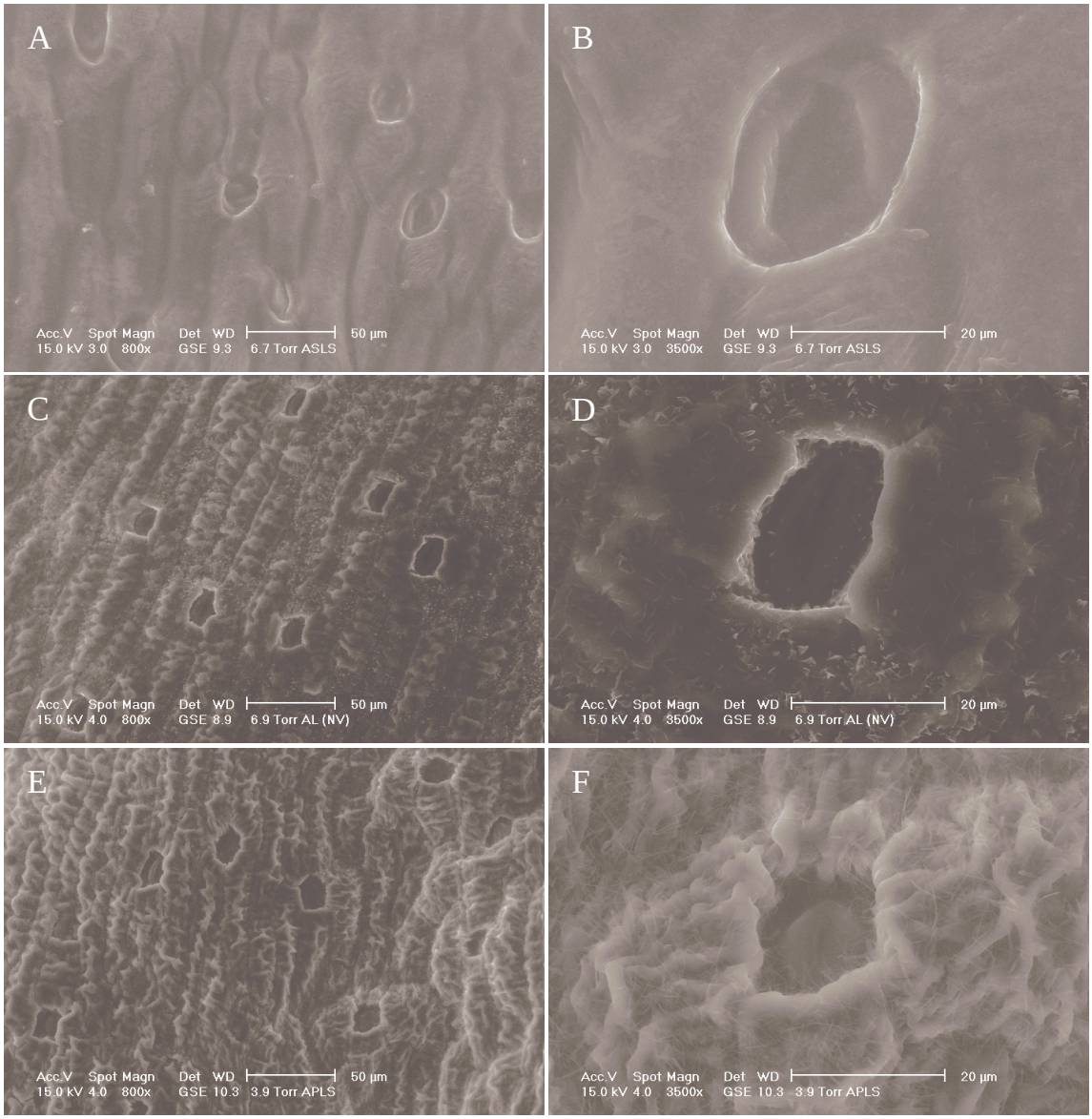

Figure 5.6: Environmental scanning electron microscopy of leaves of normal shoots of *A. polyphylla* obtained after 8 weeks of culture on media solidified with agar and different closures of the tissue culture containers: polypropylene screw on lid, sealed with Parafilm (A and B); polypropylene screw on lid only (C and D); and modified polypropylene screw on lid with a hole (d = 7 mm), covered with polyester mesh (E and F). View of the abaxial leaf surface showing the epidermis, numerous stomata and epicuticular wax.

The water loss from the culture media caused an increase of agar and gelrite concentrations (Figure 5.8). In ventilated cultures, within the 8-week culture period, the concentrations increased gradually from 8 to 14.3 g l^{-1} and from 2.4 to 5.1 g l^{-1} for agar and gelrire, respectively (Figure 5.8). Almost no increase of the gelling agent concentration was detected in media from non-ventilated containers (Figure 5.8).

5.4 Discussion

5.4.1 Effect of ventilation on hyperhydricity of A. polyphylla

In our experimental system, ventilating the culture vessels completely eliminated the occurrence of hyperhydricity (Figure 5.1, B). Similarly, ventilation was found to successfully reduce hyperhydricity in *Dianthus caryophyllus* (HAKKAART and VERSLUIJS, 1983; MAJADA *et al.* 1997; CASANOVA *et al.*, 2008), *Gypsophila paniculata* (DILLEN and BUYSENS, 1989), *Petunia hybrida* (ZIMMERMAN *et al.*, 1991), *Brassica oleracea* (ZOBAYED *et al.*, 1999b), *Citrullus lanatus* (THOMAS *et al.*, 2000), *Solanum tuberosum* (ZOBAYED *et al.*, 2001; PARK *et al.*, 2004), *Scrophularia yoshimurae* (LAI *et al.*, 2005; TSAY *et al.*, 2006) and *Thapsia garganica* (MAKUNGA *et al.*, 2006). The beneficial effects of ventilation in reducing shoot hyperhydricity may be due to a multiple interaction between: (i) reduction of the relative humidity in the vessel (MAENE and DEBERGH, 1987; BÖTTCHER *et al.*, 1988; PÉREZ-TORNERO *et al.*, 2001), (ii) increase of gas exchange with the outside atmosphere (DILLEN and BUYSENS, 1989; MAJADA *et al.*, 1997; LAI *et al.*, 2005), and (iii) decreased availability of water, plant growth regulators and mineral nutrients in the medium (DEBERGH, 1983; HAKKAART and VERSLUIJS, 1983; BORNMAN and VOGELMAN, 1984).

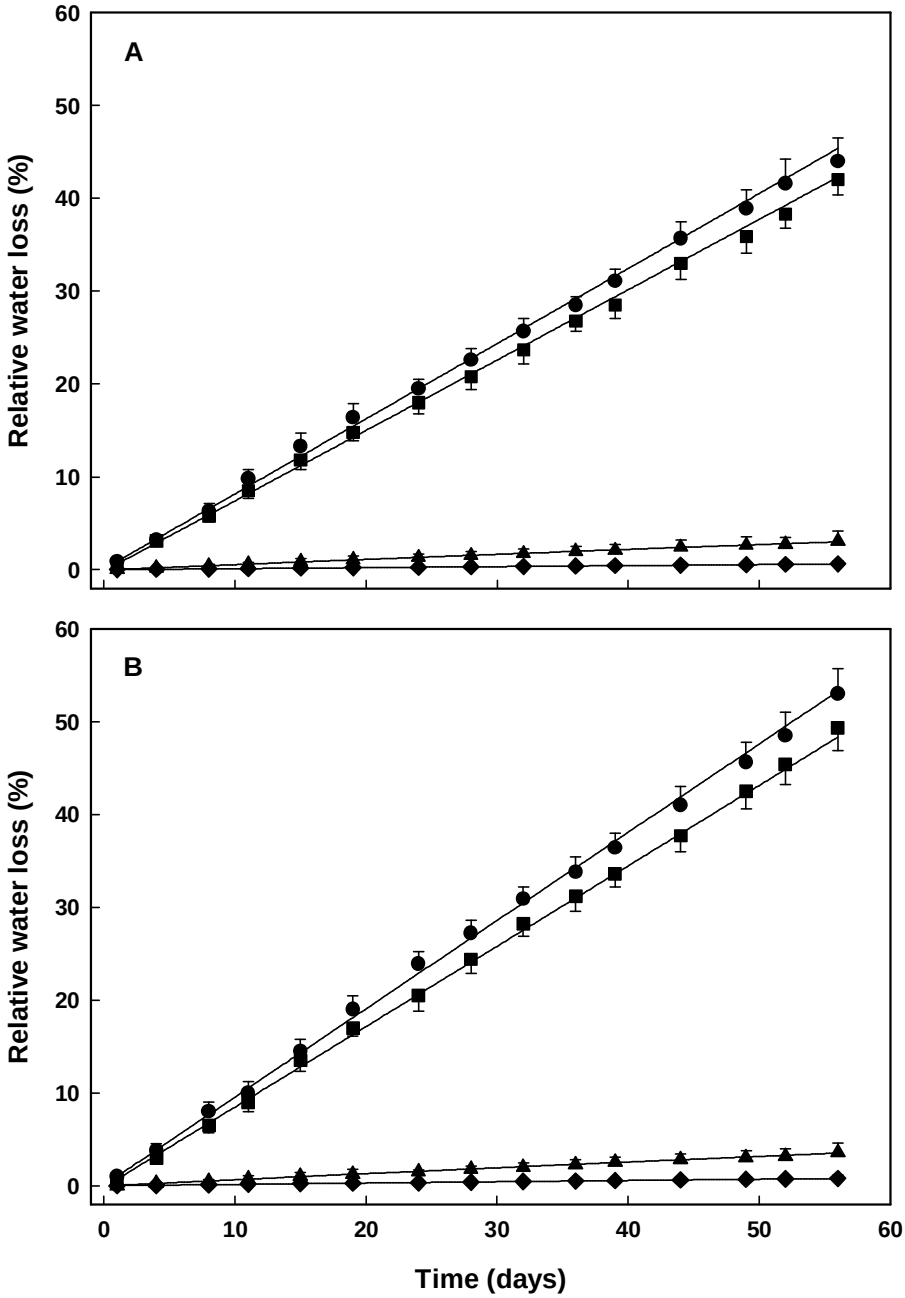

Figure 5.7: Relative water loss (%) of media, solidified with agar (A) or gelrite (B), from tissue culture containers with different closures: screw on lid, sealed with Parafilm (diamonds); screw on lid only (triangles); modified screw on lid with a hole (d = 7 mm), covered with polyester mesh (squares); and modified screw on lid with a hole (d = 7 mm), covered with cotton mesh (circles). Data points represent means ± SE (*n* = 5). Standard error bars are not visible when smaller than the symbols.

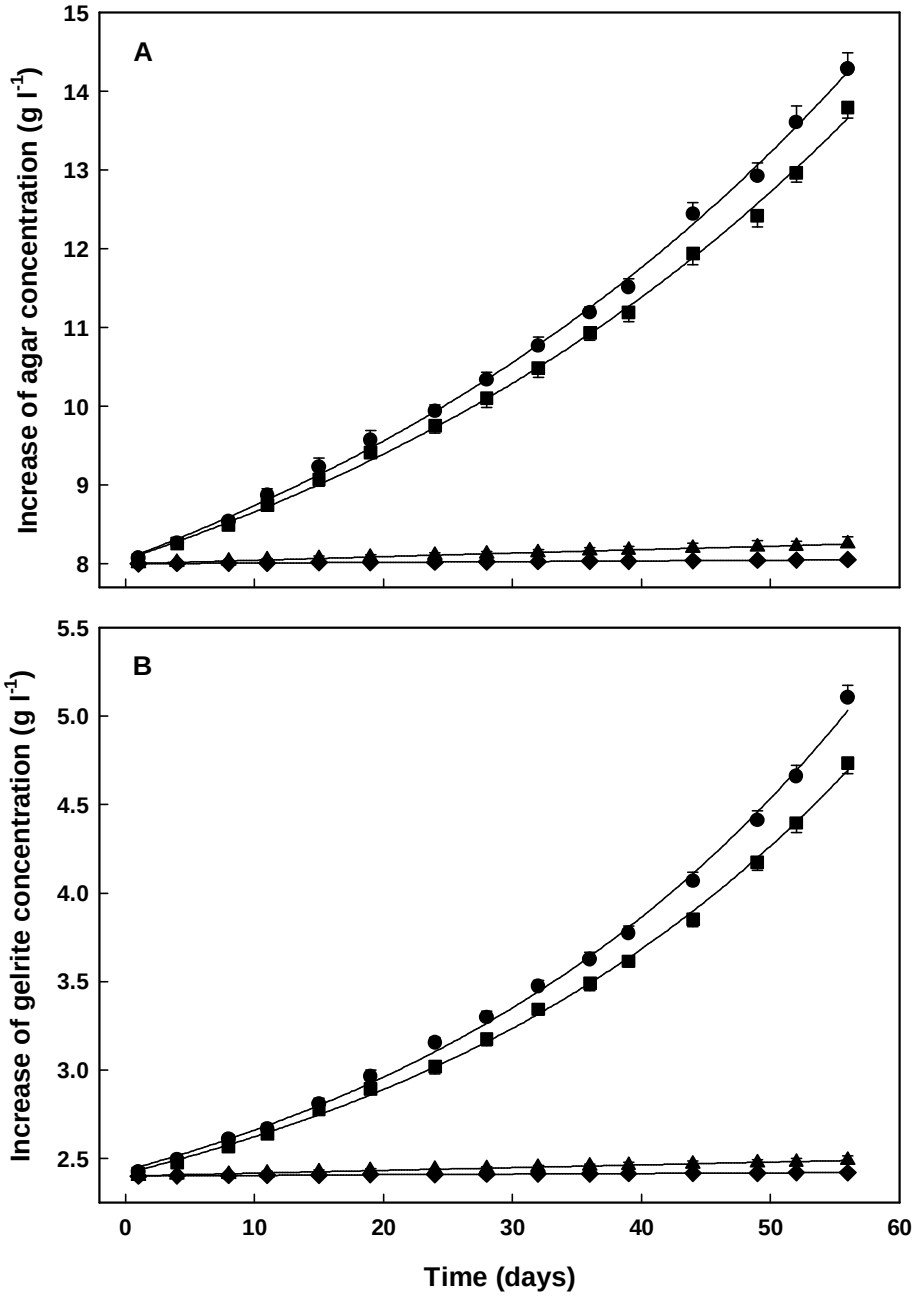

Figure 5.8: Increase of the concentration of agar (A) and gelrite (B) of media in culture vessels with different closures: screw on lid, sealed with Parafilm (diamonds); screw on lid only (triangles); modified screw on lid with a hole (d = 7 mm), covered with polyester mesh (squares); and modified screw on lid with a hole (d = 7 mm), covered with cotton mesh (circles). Data points represent means ± SE (*n* = 5). Standard error bars are not visible when smaller than the symbols.

In a previous investigation (Chapter 4), increasing the concentration of gelrite from 2.4 to 6 g l^{-1} reduced hyperhydricity to barely 51% (Figure 4.4, C). Typically, increasing the gelling agent concentration brings about reduced relative humidity in the culture container as well as decreased availability of water, mineral elements and hormone substances from the culture medium. In the present study, at the end of the 8-week culture period of ventilated cultures, the concentration of gelrite had risen to 5.1 g l^{-1} as a result of water loss of the medium through evaporation (Figure 5.8, B). Under these conditions of diffusive ventilation, hyperhydricity was entirely eliminated (Figure 5.1, B). From our results, it appears that an essential prerequisite for overcoming hyperhydricity in *in vitro* shoots of *A. polyphylla* is the removal of the accumulated gases in the headspace through increasing the gas exchange between the *in vitro* atmosphere and the outside environment. High concentrations of ethylene in closed culture vessels have been often positively correlated with hyperhydricity (KEVERS and GASPAR, 1985b; ZIV and ARIEL, 1992; LAI *et al.*, 2005). Furthermore, carbon dioxide is also regularly released in large amounts by growing tissue cultures. High CO_2 levels can stimulate ethylene biosynthesis by enhancing the activity of the enzyme that converts 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene (GRODZINSKI *et al.*, 1981). High CO_2 content may also significantly affect the transpiration and photosynthesis of the *in vitro* plants.

Non-ventilated cultures grown on agar exhibited relatively low hyperhydricity (16.8%), compared to when gelrite was used (71 – 84%; Figure 5.1, B). This may be due to the presence of components in agar that are able to control hyperhydricity (NAIRN *et al.*, 1995; see Chapter 4 for detailed discussion). Moreover, some gelling agents were shown to release ethylene in addition to the ethylene produced by explants (LEONHARDT and KANDELER, 1987), which may also account for the observed variations in hyperhydricity.

5.4.2 Effect of ventilation on shoot regeneration and growth of A. polyphylla

Improved ventilation of culture vessels enhances evapotranspiration, which alters the physical properties of the culture medium. The increase of water loss from the culture medium (Figure 5.7) leads to two major changes. The first one is the increase of agar or gelrite concentrations during the culture period (Figure 5.8), which decreases the

matric potential of the media and consequently the availability of water (see Chapter 4 for detailed discussion). The second change is the increased concentration of solutes in the medium (mineral salts, carbohydrate source, hormones), which not only decreases greatly the osmotic potential of the medium, but can be toxic to the plants (MAENE and DEBERGH, 1987; WHISH *et al.*, 1992). The decrease in the matric and osmotic potentials modifies the water potential of the culture medium, which influences *in vitro* morphogenic processes.

The reduced micropropagation rates and shoot growth in ventilated cultures of *Aloe polyphylla* (Figures 5.1, A and 5.3) could be attributed to the effect of evaporation, which alters the physical characteristics of the medium (see above) as well as the interaction between medium and explant. Other researchers have also reported that better ventilation affected negatively the number of shoots or buds formed and their length (HAKKAART and VERSLUIJS, 1983; SHORT *et al.*, 1987; SMITH *et al.*, 1990; RITCHIE *et al.*, 1991; TANAKA *et al.*, 1992b; MAJADA *et al.*, 1997; McCARTAN *et al.*, 2004; LAI *et al.*, 2005; TSAY *et al.*, 2006; CASANOVA *et al.*, 2008).

5.4.3 Effect of ventilation on chlorophyll content

Ventilation increased considerably the leaf chlorophyll content of *A. polyphylla* shoots (Figure 5.4, A). This is in agreement with other findings, where lower chlorophyll was recorded in closed vessels (COURNAC *et al.*, 1991; ZOBAYED *et al.*, 1999a, 1999b; McCARTAN *et al.*, 2004). As expected from external appearance (Figure 5.2), the chlorophyll content of hyperhydric shoots was markedly reduced compared to that of normal shoots grown under the same culture conditions (Figure 5.4, B). Lower chlorophyll was also detected in hyperhydric leaves of *Prunus avium* (PHAN and LETOUZÉ, 1983), *Eucalyptus saligna* (JONES *et al.*, 1993), and *Dianthus caryophyllus* (SAHER *et al.*, 2004). ZOBAYED *et al.* (1999b) have suggested that high ethylene concentration in non-ventilated vessels may depress chlorophyll levels. In support of this hypothesis is the finding that the addition of ethylene inhibitors ($Ag_2S_2O_3$ or $AgNO_3$) increased chlorophyll content (COURNAC *et al.*, 1991; ZOBAYED *et al.*, 1999b).

5.4.4 Effect of ventilation on leaf surface ultrastructure

Leaves of hyperhydric shoots and shoots obtained from vessels sealed with Parafilm were characterised with no wax deposition and large, open stomata (Figures 5.5 and 5.6). Such shoots have a poor survival rate when transferred to the *ex vitro* environment mainly due to water loss and desiccation (DEBERGH *et al.*, 1992). Non-functional stomata have been regarded to be the major cause of desiccation (ZIV and ARIEL, 1994). The epicuticular wax, the cuticular layer, and the opening and closing of stomata are the main barriers against plant desiccation. The failure of explants to acclimatize to the new conditions may often be a result of aberrations or malfunctioning of these barriers (DEBERGH *et al.*, 1992). Absence of epicuticular wax and stomata abnormalities have been observed in the hyperhydric leaves of various species: carnation (ZIV, 1991; ZIV and ARIEL, 1992; SATO *et al.*, 1993; OLMOS and HELLÍN, 1998), *Datura insignis* (MIGUENS *et al.*, 1993), *Eucalyptus saligna* (JONES *et al.*, 1993), pepper (FONTES *et al.*, 1999) and eggplant (PICOLI *et al.*, 2001).

The leaf surface ultrastructure of ventilated cultures revealed dense formation of epicuticular wax and smaller stomata (Figure 5.6). Diffusive ventilation of culture vessels reduced the relative humidity of the *in vitro* environment and increased the evapotranspiration rate, which led to decreased water potential of the culture medium. This favoured a microenvironment suitable for epicuticular wax formation. ZIV (1986) observed in carnation shoots an inverse correlation between the amount of surface wax and the relative humidity in the culture vessels. Lowering the headspace relative humidity in the culture containers has also been shown to improve stomatal functioning in apple (BRAINERD and FUCHIGAMI, 1981), chrysanthemum (SHORT *et al.*, 1987; SMITH *et al.*, 1990) and rose plantlets (CAPELLADES *et al.*, 1990; GHASHGHAIE *et al.*, 1992). Improving the water loss regulation mechanisms of *in vitro* plants before transplanting them to soil is beneficial for their survival.

5.5 Conclusions and Recommendations

Diffusive ventilation, achieved by simply using vessel closures with a hole covered with polyester or cotton mesh, was advantageous for *in vitro* regenerants of *Aloe polyphylla* in the following ways: (i) it completely eliminated the occurrence of hyperhydricity, (ii) increased leaf chlorophyll content, and (iii) built up the water loss regulation mechanisms of explants by increasing epicuticular wax deposition. Increased ventilation, however, affected negatively the regeneration rate and shoot growth. Future research needs to determine if the effects of ventilation are time sensitive, and perhaps a compromise between quality and quantity of the explants could be achieved by using closed vessels for the initial shoot induction and bud proliferation and ventilated containers for the subsequent stages of the *in vitro* propagation. Nevertheless, this study emphasizes the importance of ventilation to flush out gases, accumulated at high concentrations in the headspace of culture vessels, to achieve non-hyperhydric shoot morphogenesis.

Chapter 6

ENDOGENOUS CYTOKININS IN SHOOTS OF *ALOE POLYPHYLLA* CULTURED *IN VITRO* IN RELATION TO HYPERHYDRICITY, EXOGENOUS CYTOKININS AND GELLING AGENTS

6.1 Introduction

Hyperhydricity is a morphological, anatomical and physiological disorder frequently affecting *in vitro* regenerated shoots of various herbaceous and woody plant species (ZIV, 1991). This phenomenon can irreversibly develop towards the loss of tissue regeneration (GASPAR *et al.*, 2000). Hyperhydric shoots (HS) root poorly and are difficult to establish *ex vitro*. This causes losses in the commercial micropropagation industry, and limits the application of *in vitro* propagation method for conservation purposes. Leaves of HS are thick, elongated, curled (DEBERGH *et al.*, 1992) and often appear turgid, fragile, translucent and less green when compared to normal leaves (GASPAR, 1991). Anatomical characteristics change in HS, and several types of abnormal structures can be recognized (GASPAR, 1991; ZIV, 1991). At the physiological and biochemical levels hyperhydricity has been associated with altered activities of various enzymes involved in cellulose and lignin synthesis, photosynthesis, ethylene production as well as antioxidant enzymes linked with the metabolism of the activated oxygen species (KEVERS *et al.*, 1984; KEVERS and GASPAR, 1985a; FRANCK *et al.*, 1995; SAHER *et al.*, 2004). Hyperhydricity symptoms have also been correlated with changes in protein synthesis (ZIV and ARIEL 1992; PICOLI *et al.*, 2001) and even with an abnormal DNA content (OCHATT *et al.*, 2002).

Various factors have been listed as being responsible for hyperhydricity: the presence in the medium of growth regulators such as cytokinins (CKs) at supraoptimal concentrations, the type and concentration of the gelling agent, the large quantities of mineral nutrients such as NH_4^+ and Cl^- ions in the medium and

high relative humidity in the culture vessels (see GASPAR, 1991; ZIV, 1991; DEBERGH *et al.*, 1992 for reviews). The process of hyperhydricity in *in vitro* regenerants of *Aloe polyphylla* is influenced by the type of exogenous CK, its concentration and the type of gelling agent used to solidify the medium (IVANOVA and VAN STADEN, 2008, 2009). The role of exogenous CKs in the induction and promotion of hyperhydricity during *in vitro* culture of explants has been investigated in a number of studies (BORNMAN and VOGELMANN, 1984; LESHEM *et al.*, 1988; FRICK, 1991; KATAEVA *et al.*, 1991; WILLIAMS and TAJI, 1991; PICOLI *et al.*, 2001; OCHATT *et al.*, 2002; KADOTA and NIIMI, 2003). Gelrite as a gelling agent has been shown to consistently produce HS in some species, even though the media solidified with gelrite appeared to have the same gelling strength as media gelled with agar on which hyperhydricity was not induced (PASQUALETTO *et al.*, 1986, 1988a; ZIMMERMAN *et al.*, 1991; FRANCK *et al.*, 1995; IVANOVA and VAN STADEN, 2009). It has been suggested that this hyperhydricity-favouring characteristic of gelrite is related to its physical structure (WILLIAMS and TAJI, 1991).

CKs are plant hormones that influence numerous developmental and physiological processes in plants. Chemically, naturally occurring CKs are N^6-substituted adenine derivatives, and according to the substituent, three groups of CKs are recognised: isoprenoid (zeatin, iP and their derivatives), isoprenoid-derived (DHZ and its conjugates) and aromatic CKs (ZAŽÍMALOVÁ *et al.*, 1999), which include BA and its derivatives and topolins – the *meta*-topolin (*m*T), *ortho*-topolin (*o*T) and *para*-topolin (*p*T) families. These are present in the plants in various conjugated forms. Exogenously applied CKs are essential for *in vitro* shoot development (KRIKORIAN, 1995). The developmental response of explants to exogenous hormones is related to the hormone concentration in the medium. It is also a result of the hormone uptake, metabolism and transport within the explant (AUER *et al.*, 1999), which may be affected by the culture conditions (FEITO *et al.*, 1994). Growth and development *in vitro* are regulated by the interaction and balance between the plant growth regulators supplied to the medium and the growth substances produced endogenously (GEORGE, 1993). The biochemical pathways that regulate endogenous CK levels could be affected by tissue culture conditions, exogenous

plant growth regulators, genotype, explant morphology and many other factors (KRIKORIAN, 1995).

A number of reports have been published on the effect of exogenously applied CKs and auxins on the content of endogenous CKs (KATAEVA *et al.*, 1991; VANKOVA *et al.*, 1991; FEITO *et al.*, 1994, 1995; AUER *et al.*, 1999; VANDEMOORTELE *et al.*, 2001). However, information about the correlation between the endogenous CKs and hyperhydricity are limited (KATAEVA *et al.*, 1991; DANTAS de OLIVEIRA *et al.*, 1997; VANDEMOORTELE *et al.*, 2001).

The first objective of the present investigation was to identify and quantify the endogenous CKs of *in vitro* grown plantlets of *Aloe polyphylla*. We also compared the internal CK content of normal shoots (NS) and hyperhydric shoots (HS) grown under the same culture conditions. The second aim of the study was to investigate how the exogenous CKs and gelling agents affect the endogenous CK content of *in vitro* regenerants of *A. polyphylla*. We examined whether the effect of these factors on hyperhydricity in *A. polyphylla* could be mediated by changes in the endogenous CKs of the shoots.

6.2 Materials and Methods

6.2.1 Plant material and culture conditions

The shoot culture of *A. polyphylla* established from seedlings (ABRIE and VAN STADEN, 2001) were maintained and multiplied in continuous culture through a series of 8-weeks cycles. They were grown on MS medium (MURASHIGE and SKOOG, 1962) containing 5.0 µM zeatin (Sigma, St. Louis, MO, USA), 2.46 µM IBA (Sigma) and solidified with 8 g l^{-1} agar (Unilab, Saarchem, South Africa). Shoots from *in vitro*-grown plants were used as initial explants in the present study. Hyperhydricity was induced by transferring the shoots to media with different compositions. The basal medium consisted of MS salts and vitamins supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol (Sigma) and 2.46 µM IBA.

Two types of CKs [zeatin and BA (Sigma)] applied exogenously at two different concentrations (5 and 15 µM) and two types of gelling agents [8 g l^{-1} agar and 2.4 g l^{-1} gelrite (Labretoria, South Africa)] were used. The control at a particular gelling agent treatment (agar or gelrite) was without exogenous CKs. The pH of all media was adjusted to 5.8 with 1N KOH or 1N HCl before autoclaving at 121 ℃ and 105 kPa for 20 min. The explants were cultured in 250 ml tissue culture flasks, each containing 50 ml of medium and closed with polypropylene screw caps; six explants were cultured per flask. Altogether there were 10 treatment combinations, and each treatment comprised 18 replicates (explants). The cultures were incubated under a continuous photoperiod (light intensity: 35 ± 2 µmol m^{-2} s^{-1}) provided by fluorescent lamps (Osram L75W/20X cool white, USA) at 25 ± 2 ℃ a nd a relative air humidity of approximately 60%.

6.2.2 Data collection and statistical analyses

At the end of 8-weeks culture period the number of shoots per explant was recorded, and the newly-formed shoots were classified as normal shoots (NS) or hyperhydric shoots (HS), according to their external appearance (ZIV, 1991). HS were shorter with thicker, translucent leaves compared to NS, whose leaves showed no abnormality. Hyperhydricity (%) was calculated based on the above information: number of HS per explant / total number of shoots per explant × 100. Samples of NS and HS from different treatments were collected for endogenous CK analysis. Shoots were removed from the media and rinsed with distilled water to remove medium residues. The samples were freeze-dried and then stored at –70° C until their use for internal CK determination.

The hyperhydricity (%) data were transformed into arc sine square roots prior to statistical analysis. The data on multiplication rate and hyperhydricity were analysed by one-way analysis of variance (ANOVA) using Genstat 8 (Release 8.1) software. Fisher's Least Significant Difference (LSD) test was calculated to separate the means at $P \leq 0.05$.

6.2.3 Cytokinin analysis

6.2.3.1 Extraction and purification of the endogenous CKs

The procedure used for analysis of endogenous CKs was a modification of the method described by NOVÁK *et al.* (2003). Freeze-dried plant material was homogenized in liquid nitrogen to a fine powder and extracted in ice-cold 70% (v/v) ethanol. Deuterium-labelled CK internal standards (Olchemim Ltd., Czech Republic) were added, each at 5 pmol per sample to check the recovery during purification and to validate the determination. The standards were [$^2H_5$]*t*Z, [$^2H_5$]*t*ZR, [$^2H_5$]*t*Z9G, [$^2H_5$]*t*ZOG, [$^2H_5$]*t*ZROG, [$^2H_5$]*t*ZRMP, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_3$]DHZOG, [$^2H_3$]DHZROG, [$^2H_3$]DHZRMP, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP9G, [$^2H_6$]iPRMP, [$^2H_7$]BA, [$^2H_7$]BAR, [$^2H_7$]BA9G, [$^2H_7$]BARMP, [$^{15}N_4$]*m*T, and [$^{15}N_4$]*o*T. All topolins were analysed using internal deuterium standards for [$^{15}N_4$]*m*T and [$^{15}N_4$]*o*T as no other labelled standards were available. Therefore, the values of other topolin metabolites may have an error load which originates from imperfect internal standardization. After 3 h extraction, the homogenate was centrifuged (15 000 *g* at 4℃) and the pellets were re-extracted. The combined supernatants were concentrated to approximately 1.0 ml by rotary evaporation under vacuum at 35℃. The samples were diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5).

The extracts were purified using a combined DEAE-Sephadex (1.0 × 5.0 cm)-octadecylsilica (0.5 × 1.5 cm) column and immunoaffinity chromatography (IACh) based on generic CK monoclonal antibodies as described by FAISS *et al.* (1997). This resulted in three fractions: (1) the free bases, ribosides and N-glycosides (fraction B), (2) a nucleotide fraction (NT) and (3) an *O*-glucoside fraction (OG). The metabolic eluates from the IAC columns were evaporated to dryness and dissolved in 50 µl of the mobile phase used for HPLC analysis.

6.2.3.2 Identification and quantification of the endogenous CKs; HPLC-MS conditions

The samples were analysed by HPLC (Waters Alliance 2690) linked to a Micromass ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface [LC(+)ES-MS] and photodiode array detector (Waters PDA 996). Samples

were injected into a C_{18} reverse phase column (Waters, Symmetry, 5 µm, 150 mm × 2.1 mm), and elution was performed with a metabolic gradient composed of 100% methanol (A) and 15 mM formic acid (B) adjusted to pH 4.0 with ammonium under the conditions described by NOVÁK *et al.* (2003). Using a post column split of 1:1, the effluent was introduced into an electrospray source (NOVÁK *et al.*, 2003). Quantitative analysis of the different CKs was performed in selective ion recording (SIR) mode. Quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to appropriate labelled standard was determined and used further to quantify the level of endogenous compounds in the original extract, according to the known quantity of added internal standard (NOVÁK *et al.*, 2003). The calibration range was 0.05 – 100.00 pmol 25 μl^{-1} for all CKs tested. All samples were analysed in triplicate. The results presented here are the mean values of two independent experiments.

6.3 Results

6.3.1 Regeneration rate and hyperhydricity

The analysis of variance showed that variations due to various treatments involving the type of gelling agent, the type of exogenous CK and CK concentration were significant ($P \leq 0.05$) for multiplication rate (number of shoots per explant) and hyperhydricity (%). Regeneration occurred in all media combinations tested, including the media lacking exogenous CKs (Table 6.1). The highest multiplication rate was observed on media with agar and CK (zeatin or BA) applied at 5 µM. Increasing the concentration of CK to 15 µM reduced the number of shoots obtained. On media with agar very low hyperhydricity was recorded (Table 6.1). The treatments with gelrite produced NS and HS at the same time (Figure 6.1, A), although the media gelled with 2.4 g l^{-1} gelrite exhibited the same relative matric potential as with 8 g l^{-1} agar (see Chapter 4 for details). The highest hyperhydricity was observed on media with gelrite and CK (BA or zeatin) at 15 µM (Table 6.1; Figure 6.1, B). Spontaneous HS also appeared on CK-free media, and they appeared exactly the same as regular HS.

Table 6.1: Effect of gelling agent and exogenous cytokinin type and concentration on the multiplication rate (number of shoots per explant) and hyperhydricity (%) of *Aloe polyphylla* after 8 weeks in culture

Gelling agent	Exogenous CK (µM)		Multiplication rate (shoots / explant) [a]	Hyperhydricity (%) [a]
Agar	Zeatin	5.0	42.5 a	1.8 d
		15.0	24.1 b	8.0 d
	BA	5.0	42.4 a	0.6 d
		15.0	17.3 c	7.9 d
	Control	—	3.2 e	16.7 d
Gelrite	Zeatin	5.0	21.8 bc	48.4 c
		15.0	26.6 b	70.0 ab
	BA	5.0	25.0 b	54.8 bc
		15.0	10.1 d	82.9 a
	Control	—	5.2 de	15.8 d

[a] Means followed by common letters within a column are not significantly different at $P \leq 0.05$, according to least significant difference (LSD) test.

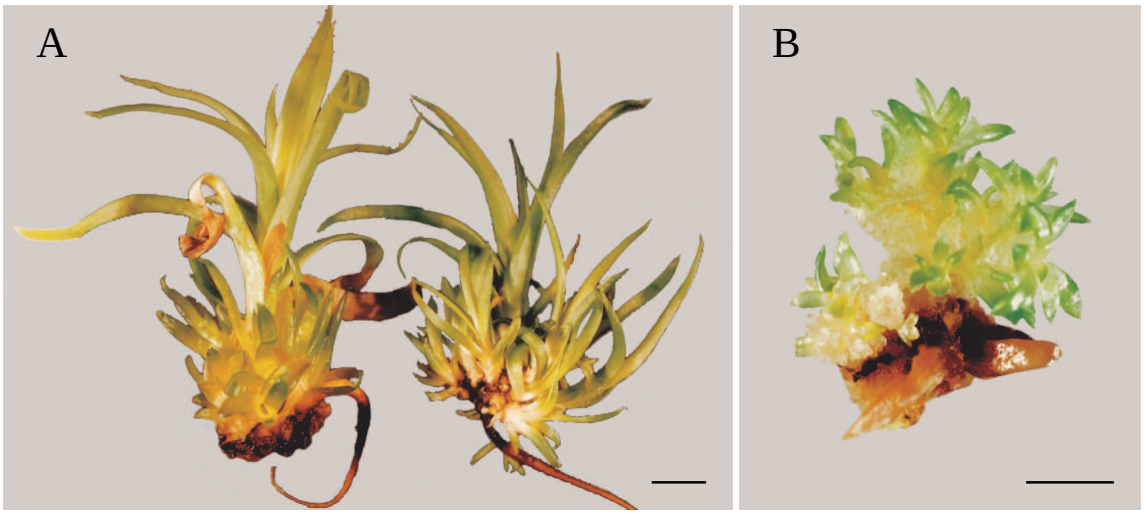

Figure 6.1: Morphology of *A. polyphylla* shoots after 8 weeks of culture. (A) Normal shoots (NS; right) and hyperhydric shoots (HS; left) obtained from medium with 5 µM zeatin and gelrite. (B) HS grown on medium supplemented with 15 µM BA and solidified with gelrite. Bar = 10 mm.

6.3.2 Endogenous CK content

Thirty two isoprenoid and aromatic CKs were identified and quantified in NS and HS of *A. polyphylla* grown in the various treatments (Table 6.2). These comprised iP, *t*Z, *c*Z, DHZ, BA and topolin derivatives. Free bases, ribosides, *O*-glucosides, N-glucosides and nucleotides were detected. Total CK content in HS was higher than that of NS grown on the same medium composition (Table 6.2). Similarly, total CK content in HS grown on medium with agar and no exogenous CKs was 3 times higher than that of NS grown on the same medium. However, when gelrite was used no significant difference in the total CK levels in HS and NS could be detected. When zeatin (5 or 15 µM) or BA (5 µM) was used as exogenous CK, HS showed 3- to 4-fold enhancement of total CK contents compared to the respective NS grown on the same media. Increasing the BA concentration in the medium to 15 µM substantially enhanced the total CK levels in HS (27-fold) as compared to the NS. The application of exogenous CKs led to an overall increase in total CK contents in the shoots compared to those grown on media without CKs (Table 6.2). Supplementing the medium with 5 and 15 µM zeatin resulted in 20-fold and 69-fold enhancement, respectively, in HS. BA at 5 and 15 µM increased the endogenous CK concentrations in HS by 72-fold and 2222-fold, respectively (Table 6.2).

The CK content presented as the sum of CKs belonging to different structural groups is shown in Figures 6.2 and 6.3. The CKs identified in the shoots grown on media without exogenous CKs comprised only iP-, *t*Z- and *c*Z-type CKs. No DHZ-type isoprenoid CKs and aromatic CKs were detected (Figure 6.2, A and B). HS contained higher levels of iP- and *t*Z-type CKs and a lower percentage of *c*Z-type as compared to NS (Figure 6.2, A and B). Application of zeatin to the culture media resulted in the formation and presence of DHZ-type CKs in the newly-formed shoots together with iP-, *t*Z- and *c*Z-type CKs. No aromatic CKs were detected (Figure 6.2, C-F). When agar was used as a gelling agent, the CK pool in NS was predominantly of the *t*Z-type followed by iP-, *c*Z- and DHZ-types (Figure 6.2, C and E). DHZ-type CKs were the dominant compounds in the CK pool of NS and HS grown on media with gelrite. HS showed very high levels of DHZ-type CKs as compared to NS (Figure 6.2, D and F).

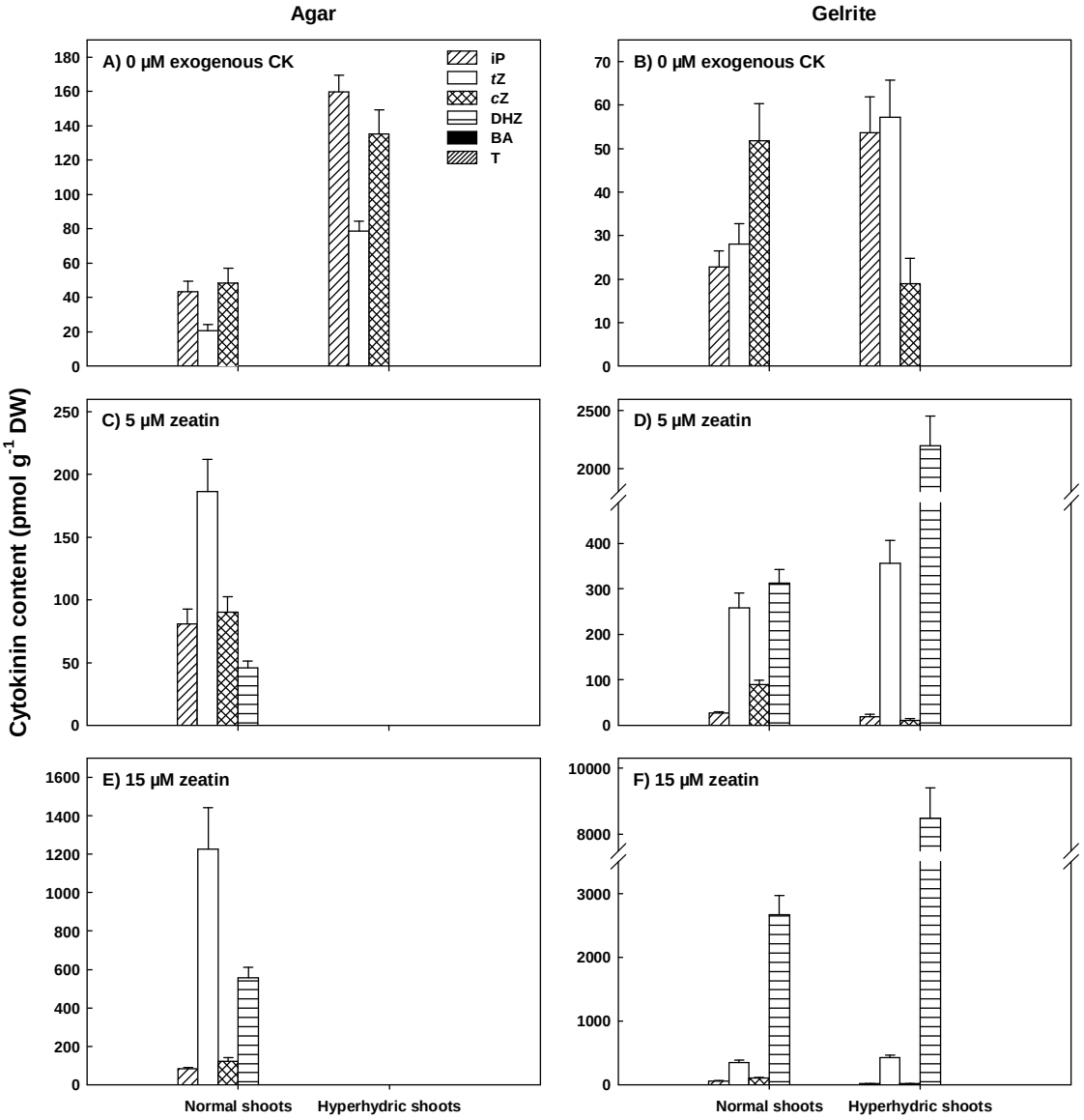

Figure 6.2: Content of structurally different cytokinin groups in normal and hyperhydric shoots of *A. polyphylla* obtained after 8 weeks of culture. Shoots were grown on media without exogenous cytokinins (A, B) or supplemented with 5 µM (C, D) and 15 µM zeatin (E, F), and solidified with either agar or gelrite. Data are means ± SE (n = 6). (BA – N^6-benzyladenine, DHZ – Dihydrozeatin, iP – N^6-(Δ^2-isopentenyl)adenine, T – Topolin, *c*Z – *cis*-zeatin, *t*Z – *trans*-zeatin).

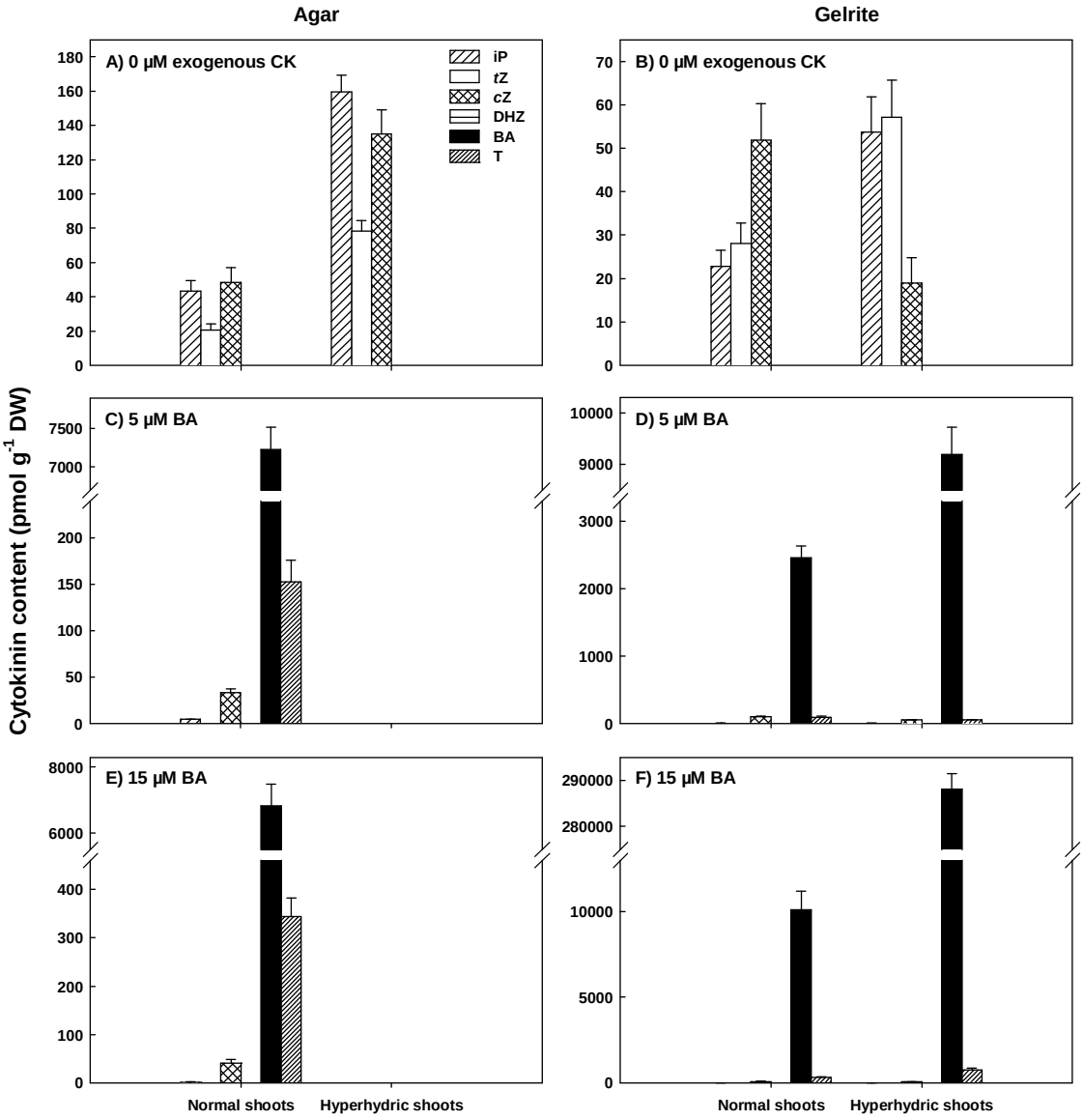

Figure 6.3: Content of structurally different cytokinin groups in normal and hyperhydric shoots of *A. polyphylla* obtained after 8 weeks of culture. Shoots were grown on media without exogenous cytokinins (A, B) or supplemented with 5 µM (C, D) and 15 µM BA (E, F), and solidified with either agar or gelrite. Data are means ± SE (n = 6). (BA – N^6-benzyladenine, DHZ – Dihydrozeatin, iP – N^6-(Δ^2-isopentenyl)adenine, T – Topolin, *c*Z – *cis*-zeatin, *t*Z – *trans*-zeatin).

Application of exogenous BA to the media resulted in a transition from isoprenoid to aromatic CKs in the newly-formed shoots compared to those grown on CK-free and zeatin-supplemented media (Figures 6.2 and 6.3). This CK pool mainly consisted of BA-type CKs, ranging from 92.5% to 99.7% in various samples from media supplemented with BA irrespective of its concentration and the gelling agent used (Figure 6.3, C-F). Very low concentrations of topolin (T)-type and *c*Z-type CKs (0.5 – 3.8%) were also detected (Figure 6.3, C-F). High concentrations of BAR and BARMP were mainly responsible for an increase in the BA-type CKs as well as total CK content of HS (Table 6.2).

iPR, iPRMP, *c*ZR and *c*ZROG were detected in all samples analysed (iPR was not found in two of the samples) irrespective of the type of exogenous CK, its concentration and the type of gelling agent used (Table 6.2).

6.4 Discussion

6.4.1 Endogenous CK content of A. polyphylla

This study is the first report on endogenous CKs in *Aloe*. Our results showed that in NS of *A. polyphylla* grown *in vitro* on CK-free medium only isoprenoid CKs were produced. The most abundant were the *c*Z-type, followed by the iP- and *t*Z-type CKs (Figure 6.2, A and B). *t*Z and its riboside are considered the predominant CKs in higher plants (McGAW and BURCH, 1995; PRINSEN *et al.*, 1997; MOK and MOK, 2001). CK *cis*-isomers are believed to be inactive or only weakly active in whole plants due to their low biological activity in CK bioassays (SCHMITZ *et al.*, 1972; VAN STADEN and DREWES, 1991), and no clear function has been assigned to them (VEACH *et al.*, 2003). However, recent analyses showed that *c*Z and its derivatives could be the dominant CKs in some organs and/or at specific stages of development in plants, such as chickpea (EMERY *et al.*, 1998), lupine (EMERY *et al.*, 2000) and maize (VEACH *et al.*, 2003), thus indicating that *cis*-isomers may have unique physiological functions (VEACH *et al.*, 2003). There are even indications that *c*Z is an active CK in *Zea mays* as three CK receptors were found to be responsive to *c*Z as well as *t*Z and iP (YONEKURA-SAKAKIBARA *et al.*, 2004). The present study has further shown that although the levels of total CKs vary markedly across the

treatments and between NS and HS, the levels of individual *cis*-isomers, particularly *c*ZR and *c*ZROG, remain relatively constant (Table 6.2), suggesting that they may have some specialized functions in *A. polyphylla*.

The origin of these levels of *cis*-forms in *A. polyphylla* is not known. One possibility is that they may be derived from the breakdown of CK-containing tRNA. Objections to this pathway are the low turnover rate of tRNA and the general nature of tRNA degradation, which is not a regulated process and occurs in all tissues, while CK biosynthesis is a highly regulated process and is localised in specific sites (MOK and MOK, 2001). Two *cis*-specific *O*-glucosyltransferases have recently been isolated from maize kernels (MARTIN *et al.*, 2001; VEACH *et al.*, 2003), suggesting the possible existence of a pathway for the metabolism of *cis*-isomers, served by *cis*-specific enzymes analogous to the *trans*-isomer pathway (MARTIN *et al.*, 2001). The distinct origins of large proportions of DMAPP (dimethylallyl diphosphate, considered as the precursor for the biosynthesis of isoprenoid CKs) for *t*Z and *c*Z biosynthesis in *Arabidopsis* suggest as well that plants are able to independently modulate the levels of *cis*- and *trans*-forms (KASAHARA *et al.*, 2004).

6.4.2 Effect of exogenous CKs on the endogenous CK content of A. polyphylla

We observed that there was a lot of variation in the types of CKs (in shoots) obtained from diverse treatments, and this was because the shoots were grown under different conditions in the present study (Table 6.2; Figures 6.2 and 6.3). It is known that there are alternative pathways for CK biosynthesis in plants (TAYLOR *et al.*, 2003), operating together and possibly varying in a tissue- and time-dependent manner in response to environmental stimuli (ÅSTOT *et al.*, 2000).

6.4.2.1 Effect of exogenous zeatin

Several reports have indicated the positive effect of exogenous CKs on the accumulation of endogenous CKs (VANKOVA *et al.*, 1991; AUER *et al.*, 1999; VANDEMOORTELE *et al.*, 2001; MOTYKA *et al.*, 2003). Shoots, grown on media with zeatin showed an increase in total CK content compared to shoots from CK-free media (Table 6.2). This increase was proportional to the concentration of zeatin

applied to the media, and indicated a high rate of CK metabolism in the shoots. CKs, predominantly of the *t*Z- and DHZ-types, were responsible for the increase. However, no significant enhancement was found in iP-type CK levels (Table 6.2), indicating either a very rapid hydroxylation of iP-type CKs to accumulating Z-type CKs (TAKEI *et al.*, 2004) or the operation of an iP-independent route for biosynthesis of Z-type CKs (ÅSTOT *et al.*, 2000). The high metabolic activity in the shoots was evident by the elevated content of *t*Z-type CKs and the tendency they showed in their turn to be reduced, yielding DHZ-type CKs (ZAŽÍMALOVÁ *et al.*, 1999).

CK oxidase could be another factor influencing the levels of CK forms in the shoots. CK oxidase is the key enzyme of CK degradation and plays a role in controlling CK levels in plant tissues. It catalyses the irreversible metabolic degradation of CKs bearing unsaturated isoprenoid side chains (ARMSTRONG, 1994; HARE and VAN STADEN, 1994). It was shown that exogenous (both substrate and non-substrate) CKs or enhanced accumulation of endogenous CKs induced an increase in CK oxidase activity (MOTYKA *et al.*, 1996, 2003; AUER *et al.*, 1999). Such an increase in the activity could explain the presence of much higher concentrations of DHZ-type CKs (which are resistant to degradation by CK oxidase; ARMSTRONG, 1994) than that of iP- (preferred substrates of CK oxidase) and *t*Z-type CKs (exhibit a low affinity to the enzyme) in shoots grown on media with zeatin and gelrite (Figure 6.2, D and F).

The increase in the concentration of CK *O*-glucosides in shoots from media with zeatin (Table 6.2) could be an important part of a mechanism regulating the level of active CKs in plant tissues (MOK and MOK, 2001). *O*-glucosides are stable storage forms (LETHAM, 1994), and can be converted into their corresponding active CKs by β-glucosidases (BRZOBOHATÝ *et al.*, 1993). An accumulation of CK nucleotides was also observed, particularly in the shoots grown on media with gelrite (Table 6.2), thus suggesting that CK biosynthesis was activated under these conditions (SAKAKIBARA and TAKEI, 2002). The increase in the total endogenous CKs in the shoots grown on CK-media (Table 6.2) could be the result of CK production by the shoot apical meristems, which may well be the site of CK biosynthesis due to the presence of actively dividing cells (LETHAM, 1994).

6.4.2.2 Effect of exogenous BA

Shoots, from media with exogenous BA also demonstrated an overall increase in the total CK content compared to shoots grown on CK-free media (Table 6.2). Furthermore, a shift away from isoprenoid to aromatic CKs was detected and the CK pool of the shoots was comprised predominantly of BA-type, followed by topolin-type CKs (Figure 6.3, C-F). Ours is the first report showing such a distinct transition from isoprenoid to aromatic CKs in the endogenous CK content in response to BA supplemented in the media. Isoprenoid and aromatic CKs have overlapping spectra of biological activity (STRNAD, 1997). However, they are not considered as being merely alternative forms of the same signal due to the differences in their biosynthetic pathways and the nature of their receptors (STRNAD, 1997).

Aromatic CKs have been identified in various plant tissues (summarized in TAYLOR *et al.*, 2003). BA derivatives were also detected in plants as products of exogenous BA metabolism and/or uptake from culture medium (reviewed in VAN STADEN and CROUCH, 1996). The biosynthetic pathway for aromatic CKs is unknown (MOK and MOK, 2001), and the structure of the aromatic side chain suggests that it is considerably different from that proposed for the isoprenoid CKs (VAN STADEN and CROUCH, 1996; STRNAD, 1997; MOK and MOK, 2001). Several compounds have been proposed as possible precursors for aromatic CKs, namely phenolics (STRNAD, 1997), the amino acid phenylalanine (ZAŽÍMALOVÁ *et al.*, 1999) and a monoterpenoid (TAYLOR *et al.*, 2003). However, there is no experimental evidence to support these assumptions (TAYLOR *et al.*, 2003). Interconversions between the BA metabolites detected in the shoots (Table 6.2) involve enzymes common to purine metabolism, which are not strictly specific with respect to the N^6-side chain configuration (VAN STADEN and CROUCH, 1996; MOK and MOK, 2001).

Topolin-type CKs detected in the shoots comprised only a small part of the CK pool (Figure 6.3, C-F). KAMÍNEK *et al.* (1987) proposed that position-specific hydroxylation of the aromatic side chain might represent a mechanism of regulating the activity of the aromatic CKs. If so, an increase or decrease in CK activity is expected to result from hydroxylation in *meta*-position or *ortho*-/ *para*-position, respectively. However, in the present study deactivation of the endogenous aromatic

CKs (BA-type) by *ortho*- and *para*-hydroxylation was not observed as evident from the low concentrations of *o*T- and *p*T-type CKs detected in the shoots (Table 6.2).

6.4.3 Effect of gelling agent on the endogenous CK content of A. polyphylla

The capacity of gelrite to consistently give rise to hyperhydricity (Table 6.1) has been related to its physical structure. Although the media solidified with gelrite exhibited the same relative matric potential as agar media (see Chapter 4), on which hyperhydricity was very low, the physical structure of gelrite appears to allow an increased absorption by the shoots of substances, such as CKs, NH_4^+ and water suspected to be responsible of hyperhydricity (WILLIAMS and TAJI, 1991). We also observed comparatively high levels of endogenous CKs in shoots (both NS and HS) grown in gelrite media (Table 6.2). And this is in agreement with the above hypothesis indicating the occurrence of higher CK uptake and/or metabolism in shoots grown on gelrite media. The observed effect of agar on hyperhydricity has been suggested to be due to a sulphated galactan in agar (NAIRN *et al.*, 1995), which is able to prevent the symptoms of hyperhydricity.

6.4.4 Hyperhydricity and endogenous CKs

KATAEVA *et al.* (1991) postulated that hyperhydricity could be related to increased levels of endogenous CKs in the explants. Our results showed that in the various treatments the total CK content in HS was always higher than that of NS grown on the same medium composition (Table 6.2). This apparent correlation between hyperhydricity and endogenous CK levels is consistent with previous data (KATAEVA *et al.*, 1991; DANTAS de OLIVEIRA *et al.*, 1997; VANDEMOORTELE *et al.*, 2001). The general increase in internal CKs could indicate a higher CK metabolism in HS. This elevated CK content may be a result of an overproduction of CKs and/or their over-accumulation as a consequence of a defect in CK signalling. However, although the occurrence of hyperhydricity correlated with increased endogenous CKs, a causal relationship could not be established. This suggests that an increase in the endogenous level of CKs cannot be sufficient to induce hyperhydricity. Some NS across the treatments (for example, NS from media with gelrite and 15 µM zeatin or BA; Table 6.2) did not show any hyperhydricity, although they had about the same or

higher levels of CKs than that of HS from other treatments (for example, HS from media with gelrite and 5 µM zeatin or BA; Table 6.2). This is in favour of the hypothesis that hyperhydricity is a multifactor process in which CKs definitely play an important role.

6.5 Conclusions

In summary, the analysis of the endogenous CK content of *A. polyphylla* shoots revealed the following: (i) thirty-two isoprenoid and aromatic CKs were detected in the various treatments; (ii) the natural CK pool of plants from CK-free medium comprised only iP-, *t*Z and *c*Z-type CKs; (iii) application of zeatin in the medium resulted in the detection of DHZ-type CKs in the shoots; but (iv) addition of BA to the medium led to a transition from isoprenoid CKs to aromatic Cks in the respective plants; (v) as expected, the application of exogenous CKs resulted in an elevation of the internal CK levels; (vi) shoots grown on gelrite exhibited higher CK content than shoots obtained from agar; (vii) hyperhydric shoots always displayed higher CK content than normal shoots grown on the same medium composition. Our results suggest that the ability of exogenous CKs and gelrite to induce hyperhydricity in *in vitro* culture of *A. polyphylla* is associated with up-regulation of the endogenous cytokinins.

6.6 GENERAL CONCLUSIONS

Aloe polyphylla, a member of the Asphodelaceae, has been listed as highly endangered in the Red Data List due to its use for ornamental and medicinal purposes. *In vitro* propagation has proven to be a valuable method for the conservation of this species by producing large numbers of plants in a short time. However, the *in vitro* culture was hindered by the occurrence of hyperhydricity. During the process of hyperhydricity plants undergo detrimental changes at morphological, anatomical and physiological levels. Leaves of hyperhydric *A. polyphylla* plants were water-soaked, thick and translucent, with decreased chlorophyll content, no epicuticular wax deposition and large, open stomata. Typically, hyperhydric plants gradually lose their regeneration ability *in vitro* and

exhibit a low survival rate when transplanted to the *ex vitro* environment due to poor development of the rooting system and underdeveloped mechanisms for regulation of water loss. This causes significant financial losses to the micropropagation industry and limits the application of the *in vitro* propagation method. In the present research, the first principal objective was to understand the role of various culture factors involved in the process of hyperhydricity in *in vitro* regenerants of *A. polyphylla*. Several factors were identified as being responsible: high concentrations of cytokinins in combination with elevated NH_4NO_3 levels, high ratio of NH_4^+ : NO_3^- ions, liquid media, low matric potential of the media, the type of gelling agent and high relative humidity in the culture vessels. Secondly, we identified the *in vitro* conditions, under which this disorder could be successfully reduced: use of zeatin and N^6-benzyladenine at concentration of 5.0 µM, the presence of no more then 10 mM NH_4NO_3 in the medium or substituting NH_4NO_3 with nitrate or glutamine as the sole source of nitrogen at concentrations of 30 – 60 mM and 5.0 g l^{-1}, respectively, increased levels of gelrite, use of agar as a gelling agent, and use of ventilated tissue culture containers allowing gaseous exchange between the *in vitro* atmosphere and the outside environment. Finally, we investigated if the induction of this multifactor process was mediated through internal cytokinins. Although a causal relationship could not be proven, we suggest that hyperhydricity in shoots of *A. polyphylla* is at least partially due to up-regulation of endogenous cytokinin levels. Identifying the factors involved in the induction and prevention of hyperhydricity are mandatory steps in the successful use of the *in vitro* technique for the conservation of this species. Furthermore, investigating the underlying mechanisms provided new insights into our understanding of this elusive phenomenon.

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Table 6.2: Endogenous cytokinin levels in normal shoots (NS) and hyperhydric shoots (HS) of *A. polyphylla* obtained from various treatments after 8 weeks of culture

	Cytokinin content (pmol g^{-1} DW)															
	0 µM exogenous cytokinin				5 µM zeatin			15 µM zeatin			5µM BA			15 µM BA		
	Agar		Gelrite		Agar	Gelrite		Agar	Gelrite		Agar	Gelrite		Agar	Gelrite	
Cytokinin	NS	HS	NS	HS	NS	NS	HS	NS	NS	HS	NS	NS	HS	NS	NS	HS
iP	nd	nd	nd	nd	nd	nd	nd	**34.3±4.3**	**18.2±2.9**	nd	nd	nd	nd	Nd	nd	nd
iPR	**36.1±3.8**	**145±8**	**16.4±2.6**	**31.4±4.7**	**73.5±9.4**	**22.8±1.2**	**8.6±2.4**	**37.6±1.8**	**31.3±4.2**	**0.7±0.2**	**0.7±0.0**	**0.8±0.1**	**0.2±0.1**	Nd	**0.1±0.0**	nd
iPRMP	**7.3±2.2**	14.5±1.8	**6.4±1.1**	**22.3±3.5**	**7.5±2.1**	5.0±0.9	**11.1±2.3**	9.7±1.5	9.0±2.3	11.5±3.7	**3.7±0.1**	**4.2±1.2**	**4.1±0.8**	**1.6±0.3**	**2.7±0.4**	**5.3±0.9**
*t*Z	**3.8±0.5**	**7.3±0.7**	**4.3±0.2**	**5.2±0.9**	**23.8±4.3**	**21.4±3.7**	**25.3±3.9**	**355±67**	**104±9**	**109±14**	nd	nd	nd	Nd	nd	nd
*t*ZR	**11.6±2.1**	**58.8±4.3**	**11.6±2.4**	**30.2±4.5**	**74.4±6.5**	**84.4±7.8**	**42.8±3.6**	**171±32**	**64.3±9.4**	**75.9±6.6**	nd	nd	nd	Nd	nd	nd
Z9G	nd	nd	nd	nd	nd	nd	nd	**7.4±2.1**	3.0±1.2	**4.2±0.6**	nd	nd	nd	Nd	nd	nd
*t*ZOG	**0.3±0.1**	**0.4±0.1**	**3.6±0.8**	**4.4±1.2**	**13.8±3.7**	**16.3±2.6**	**22.2±4.8**	**267±36**	**41.7±5.2**	**80.7±3.7**	nd	nd	nd	Nd	nd	nd
*t*ZROG	**5.1±0.6**	**12.0±0.9**	**8.6±1.3**	**17.4±1.9**	**35.8±5.7**	**64.5±9.2**	**39.9±5.6**	**318±49**	**60.5±8.3**	**94.2±12.5**	nd	nd	nd	Nd	nd	nd
*t*ZRMP	nd	nd	nd	nd	38.5±5.6	**71.9±8.9**	**226±32**	**110±27**	**70.4±8.5**	**60.4±4.3**	nd	nd	nd	Nd	nd	nd
*c*ZR	**26.2±5.3**	**13.9±2.1**	**16.6±2.1**	**10.2±1.6**	**39.8±5.3**	**21.2±2.7**	**5.8±3.6**	**37.2±5.7**	**37.0±6.1**	**7.0±0.4**	**18.6±2.2**	**36.1±2.8**	**13.5±0.9**	**7.8±1.3**	**21.3±2.6**	**21.1±1.7**
*c*ZOG	nd	2.5±0.4	nd	nd	nd	nd	nd	0.8±0.2	nd	0.4±0.1	nd	nd	nd	Nd	nd	nd
*c*ZROG	**22.1±3.5**	**89.0±7.6**	**17.3±3.8**	**8.7±4.3**	**20.4±3.5**	**20.2±1.1**	**4.7±0.7**	**38.6±5.8**	**18.0±2.6**	**5.5±3.1**	**14.9±1.3**	**31.5±4.8**	**10.0±2.1**	**9.1±1.1**	**23.7±2.6**	**14.2±0.8**
*c*ZRMP	nd	29.7±3.9	17.9±2.6	nd	29.9±3.5	**48.1±5.6**	nd	**44.7±6.4**	**46.3±4.3**	nd	nd	34.4±2.9	27.6±3.1	**24.2±4.8**	39.2±4.2	21.8±3.7
DHZ	nd	nd	nd	nd	**4.4±0.2**	**11.1±2.3**	**32.6±2.7**	**32.6±5.3**	**85.9±18.0**	**160±45**	nd	nd	nd	Nd	nd	nd
DHZR	nd	nd	nd	nd	**20.0±3.2**	**89.3±9.7**	**590±68**	**157±13**	**630±72**	**1618±312**	nd	nd	nd	Nd	nd	nd
DHZ9G	nd	nd	nd	nd	nd	nd	3.2±0.6	3.5±0.8	**11.4±2.3**	**20.3±3.7**	nd	nd	nd	Nd	nd	nd
DHZOG	nd	nd	nd	nd	**0.6±0.1**	**5.0±0.3**	**33.3±4.1**	**25.1±2.2**	**33.9±5.7**	**228±26**	nd	nd	nd	Nd	nd	nd
DHZROG	nd	nd	nd	nd	**21.0±1.8**	**95.0±8.6**	**357±42**	**213±19**	**713±82**	**3731±342**	nd	nd	nd	Nd	nd	nd
DHZRMP	nd	nd	nd	nd	nd	**113±9**	**1178±145**	**124±15**	**1189±128**	**2732±189**	nd	nd	nd	Nd	nd	nd
BA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**1030±120**	**640±72**	**742±82**	**1605±180**	**2777±530**	**7551±615**
BAR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**1053±98**	**584±63**	**4268±372**	**782±91**	**1615±112**	**202405±1637**
BA9G	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**155±18**	22.5±4.6	**252±33**	**356±42**	**158±9**	**3603±215**
BARMP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**4993±54**	**1214±28**	**3938±43**	**4075±352**	**5568±416**	**74498±853**
*m*T	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	11.7±2.7	nd	nd	10.0±0.8	13.7±2.1	nd
*m*TR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	13.3±3.1	12.7±4.5	6.4±1.2	**35.2±4.7**	27.5±3.0	**193±21**
*m*TOG	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	32.3±8.6	18.2±3.7	21.7±1.8	24.6±1.5	43.2±6.8	77.7±9.1
*m*TROG	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**64.9±6.7**	**41.6±5.3**	14.2±1.6	**178±21**	**161±19**	**260±32**
*m*TRMP	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	45.9±5.5	nd	nd
*o*T	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**30.5±2.3**	**21.2±1.1**	7.7±0.8	**50.3±4.8**	**62.3±5.6**	20.8±1.2
*p*TR	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	**73.5±3.7**
*p*TOG	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	**49.3±5.2**
*p*TROG	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd	nd	**93.2±5.8**
Total CK	113±18	373±30 3-fold[a]	103±17	130±23	403±55	689±74	2581±321 4-fold[a] 20-fold[b]	1987±294	3167±372	8939±967 3-fold[a] 69-fold[b]	7422±317	2661±194	9305±542 4-fold[a] 72-fold[b]	7205±711	10513±1113	288887±3405 27-fold[a] 2222-fold[b]

Concentrations highlighted in bold show that the measurement was in the linear part of the calibration range. No iP9G, *c*Z, *m*T9G, *o*TR, *o*T9G, *o*TOG, *o*TROG, *o*TRMP, *p*T and *p*TRMP were detected in any of the samples analysed. The data represent the means ± SE of two experiments. nd, not detected.

[a] Increase in total CK content of HS compared to NS grown on the same media composition

[b] Increase in total CK content of HS grown on media supplemented with exogenous Z or BA (5 or 15 µM) and solidified with gelrite compared to HS grown on medium with no CKs and solidified with gelrite