In vitro techniques for the improvement of growth and secondary metabolite production in *Eucomis autumnalis* subspecies *autumnalis*

By

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Submitted in fulfilment of the requirements for the degree of

Master of Science

Research Centre for Plant Growth and Development
School of Life Sciences
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Pietermaritzburg, South Africa

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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 College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

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SUPERVISOR: PROFESSOR J. VAN STADEN

_____

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Publications from this Thesis

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Conference Contribution from this Thesis

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College of Agriculture, Engineering and Science Declaration 2 - Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

Contributions: NAM performed the literature search and drafted the manuscript under guidance and supervision of JVS and JFF.

Publication 2

Contributions: All experimental work and draft manuscript were done by NAM. AOA assisted with the experimental design. JVS and JFF supervised the whole study and edited the manuscript before submission.

Publication 3

Contributions: All experimental work and draft manuscript were done by NAM. AOA assisted with the experimental design. JVS and JFF supervised the whole study and edited the manuscript before submission.

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CMC Feb 2012

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List of Abbreviations

2,4-D	2,4–Dichlorophenoxy acetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
ATM	African Traditional Medicine
BA	6-Benzyladenine
BHT	Butylated hydroxytoluene
CCE	Cyanidin chloride equivalents
CE	Catechin equivalents
CK	Cytokinin
CKX	Cytokinin oxidase/dehydrogenase
COX	Cyclooxygenase
DCM	Dichloromethane
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
ES	Explant source
Folin-C	Folin-Ciocalteu
FW	Fresh weight
GA	Gelling agent
GA_3	Gibberellic acid
GA_{4+7}	GA_4 and GA_7 gibberellin mixture
GAE	Gallic acid equivalents
HE	Harpagoside equivalents
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
INCYDE	2-Chloro-6-(3-methoxyphenyl)aminopurine
iP	N^6-Isopentenyladenine
IUCN	International Union for the Conservation of Nature
KAR_1	Karrikinolide
LDB	Leaf explant derived from primary bulb regenerants
LDL	Leaf explant derived from primary leaf regenerants

MeJa	Methyl jasmonate
Me*m*T	*meta*-Methoxytopolin or 6-(3-methoxybenzylamino)purine
Me*m*TTHP	*meta*-Methoxy 9- tetrahydropyran-2-yl topolin or 2- [6-(3 Methoxybenzylamino)-9-(tetrahydropyran-2-yl)purine
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Murashige and Skoog medium
*m*T	*meta*-Topolin
*m*TTHP	*meta*-Topolin tetrahydropyran-2-yl or 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine
NAA	α-Naphthalene acetic acid
NSAIDs	Non-steroidal anti-inflammatory drugs
PAA	Phenylacetic acid
PBZ	Paclobutrazol
PGR	Plant growth regulator
PI-55	6-(2-hydroxy-3-methylbenzylamino)purine
PPF	Photosynthetic photon flux
PTC	Plant tissue culture
SW	Smoke-water
TPA	12-O-tetradecanoylphorbol 13-acetate

Abstract

The wide utilization and popularity of medicinal plants in African Traditional Medicine (ATM) has been recognized and attributed to the effectiveness, affordability and accessibility of these medicinal plants. However, the extensive exploitation of medicinal plants has exacerbated the strain on the wild populations. *In vitro* propagation/micropropagation is an effective method which allows for mass production or multiplication of pathogen-free plants that are morphologically and genetically identical to the parent plant. In addition, the technique is contributing to the understanding of metabolic pathways and regulating the production of plant secondary products.

Eucomis autumnalis (Mill.) Chitt. subspecies *autumnalis* (Hyacinthaceae) is a valuable medicinal species in ATM and commonly traded in the urban street markets of South Africa. Currently, the conservation status of this species has not been evaluated. However, as with most bulbous plants, the wild population is continuously under threat due to over-harvesting and habitat loss via various anthropogenic factors. Thus, *in vitro* propagation is a viable means of ensuring conservation of the plant species. However, mass propagation of medicinal plants should be accompanied with increased secondary metabolite production to guarantee their therapeutic efficacy. Therefore, the current study was aimed at understanding the different factors that affect the growth and secondary metabolite production in micropropagated *E*. *autumnalis* subspecies *autumnalis*.

The influence of the type of gelling agent (gelrite versus agar) and source of initial/primary explant source (LDL = leaf explant derived from primary leaf regenerants and LDB = leaf explant derived from primary bulb regenerants) were evaluated. Gelrite-solidified medium significantly improved shoot proliferation when compared to the use of agar as a solidifying medium. In contrast, quantified phytochemicals such as flavonoids and phenolics were more enhanced in agar-supplemented media. On the basis of the explant source, shoot proliferation and secondary metabolites in regenerants from LDB were similar to those from LDL in most cases. Overall, the type of gelling agents and primary explant source individually or/and interactively significantly influenced the growth parameters as well as the production of iridoid, condensed tannin, flavonoid and phenolic content.

The influence of different types of plant growth regulators (PGRs) on growth, phytochemical and antioxidant properties were evaluated. The PGRs were BA (benzyladenine); *m*T (*meta*-topolin); *m*TTHP [*meta*-topolin tetrahydropyran-2-yl or 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine]; Me*m*T [*meta*-methoxytopolin or 6-(3-methoxybenzylamino)purine]; Me*m*TTHP [*meta*-methoxy 9-tetrahydropyran-2-yl topolin or 2-[6-(3-Methoxybenzylamino)-9-(tetrahydropyran-2-yl)purine] and NAA (α-naphthalene acetic acid). Five cytokinins (CKs) at 2 µM in combination with varying (0, 2.5, 5, 10, 15 µM) concentrations of NAA were tested. After 10 weeks of *in vitro* growth, the regenerants were acclimatized in the greenhouse for four months. Growth, phytochemical content and antioxidant activity of *in vitro* regenerants and *ex vitro*-acclimatized plants were evaluated. The highest number of shoots (approximately 9 shoots/explant) were observed with 15 µM NAA alone or with BA treatment.

Acclimatized plants derived from the 15 µM NAA treatment had the highest number of roots, largest leaf area and widest bulb diameter. While applied PGRs increased the iridoids and condensed tannins in the *in vitro* regenerants, total phenolics and flavonoids were higher in the PGR-free treatment. In contrast to the PGR-free regenerants, 5 µM NAA and 2 µM BA treatments produced the highest antioxidant activity in the DPPH (55%) and *beta*-carotene (87%) test systems, respectively. A remarkable carry-over effect of the PGRs was noticeable on the phytochemical levels and antioxidant activity of the 4-month-old plants. In addition to the development of an optimized micropropagation protocol, manipulating the type and concentration of applied PGRs may serve as an alternative approach to regulate phytochemical production in *Eucomis autumnalis* subspecies *autumnalis*.

The influence of smoke-water (SW), karrikinolide (KAR_1) and CK analogues (PI-55 = 6-(2-hydroxy-3-methylbenzylamino)purine and INCYDE= inhibitor of cytokinin dehydrogenase or 2-chloro-6-(3-methoxyphenyl)aminopurine) individually or in combination with some selected PGRs [BA (4 µM), NAA (5 µM) and both] for *in vitro* propagated *E*. *autumnalis* subspecies *autumnalis* was evaluated. While these compounds had no significant stimulatory effect on shoot proliferation, they influenced root length at varying concentrations and when interacted with applied PGRs. The longest roots were observed in SW (1:1500), PI-55 and INCYDE (0.01 µM) treatments. There was an increase in the concentration of quantified phytochemicals (especially condensed tannins, flavonoids and phenolics) with the use of these compounds alone or when combined with PGRs. In the presence of BA, an increase in the concentration of PI-55 significantly enhanced the condensed tannin, flavonoid and phenolic contents

in the regenerants. Both phenolic and flavonoid content in *E*. *autumnalis* subspecies *autumnalis* were significantly enhanced with 0.01 µM INCYDE. Condensed tannins was about 8-fold higher in 10^{-7} M KAR_1 with BA and NAA treatment when compared to the control. To some varying degree, the effect of the tested compounds on the antioxidant activity of the *in vitro* regenerants was also noticeable. In most cases, there was no direct relationship between the level of phytochemicals and antioxidant activity recorded. The current findings indicate the array of physiological processes influenced by SW and KAR_1 during micropropagation. In addition, targeting or manipulation of phytohormone metabolic pathways using CK analogues demonstrated some noteworthy effects. Perhaps, it may offer other potential practical applications in plant biotechnology and agriculture. Thus, more studies such as quantification of endogenous hormones and identification of specific phytochemicals responsible for the bioactivity in this species will provide better insights on the mechanism of action for CK analogues as well as SW and KAR_1.

Chapter 1: General introduction

1.1. Importance of plants in African Traditional Medicine

Globally, there is increasing demand for plant species due to their medicinal, horticultural and ornamental values. The wide utilization and popularity of plants in traditional medicine is often attributed to their affordability, accessibility and perceived efficacy **(MANDER 1998)**. In South Africa alone, approximately 3000 plant species are utilized for various ailments **(VAN WYK et al. 1997)** with more than 25% of these medicinal plants used by Zulu traditional healers in KwaZulu-Natal province **(HUTCHINGS et al. 1996)**. From these, approximately 350 species are extensively traded in large quantities at informal markets **(VAN WYK et al. 1997)**. An estimated 35 000 to 70 000 tonnes (worth approximately US$ 75 to 150 million) of plant materials are consumed annually **(MANDER and LE BRETON 2006)**. The excessive exploitation of medicinal plants has put a strain on the wild populations and caused about 200 species to be listed as threatened in the South African Red Data List **(RAIMONDO et al. 2009)**. This indiscriminate harvesting and over-exploitation of the natural flora has become of great concern particularly because it frequently involves the destructive harvesting of the non-renewable parts such as bulbs, rhizomes and bark in 85% of the medicinal plants **(MANDER 1998; JÄGER and VAN STADEN 2000)**. Therefore, without an effective conservation mechanism, intense harvesting of slow-growing plants can potentially lead to their extinction.

1.2. Application of micropropagation in conservation of medicinal plants

In addition to its role in the advancement of basic plant science research, micropropagation is contributing enormously to food security, crop improvement, production of secondary products for the pharmaceutical industry and conservation of endangered species **(DiCOSMO and MISAWA 1995; TAYLOR and VAN STADEN 2002a; MOYO et al. 2011; AREMU et al. 2012a; AMOO and VAN STADEN 2013a; b)**. As a key aspect of pant biotechnology, micropropagation techniques have evolved significantly over the last few decades **(VASIL 2008)**. Some of the techniques includes direct and indirect organogenesis, suspension culture and somatic embryogenesis **(STEWARD et al. 1958; PIERIK 1987; THORPE 1990)**. Generally, micropropagation involves the aseptic manipulation of excised plant tissues (explants) which are cultured under heterotrophic conditions on artificial basal media with supplements such as plant growth regulators (PGRs) and vitamins **(GEORGE 1993)**. However, the success of micropropagation of plant species depends on the intricate and often complex interactions of several factors **(GEORGE 1993; WERBROUCK 2010)**. According to **MURASHIGE (1974)**, these factors are divided into the chemical composition and physical qualities of the medium as well as the culture environment. As important chemical components, PGRs are required in culture media to stimulate and regulate various physiological and developmental processes **(NORDSTRÖM et al. 2004)**. The most commonly used PGR in micropropagation are auxins and cytokinins (CKs). The function of these aforementioned PGRs are essential and well-known **(BAJGUZ and PIOTROWSKA 2009)**. Although it is often a complex web of signal interactions, the

existence of synergistic, antagonistic and additive interactions between these groups of PGRs are well-recognized **(COENEN and LOMAX 1997)**.

In micropropagation, shoot proliferation largely depends on the type and concentration of exogenously applied CKs. Even though benzyladenine (BA) is usually the most commonly used CK in micropropagation due to its low-cost, hydroxylated analogues of BA named 6-(3-hydroxybenzyl)adenine (*meta*-Topolin, *m*T) and its derivatives have been demonstrated as (better) alternative CKs in recent times **(AREMU et al. 2012b)**. As highlighted by the authors, *m*T and its derivatives are effective growth regulators and play a significant role in the alleviation of various physiological disorders in several species.

Lately, the potential of some compounds such as 2-chloro-6-(3-methoxyphenyl) aminopurine (inhibitor of cytokinin degradation = INCYDE), 6-(2-hydroxy-3-methylbenzylamino) purine (PI-55), smoke-water (SW) and karrikinolide (KAR_1) in improving growth and phytochemical content during plant propagation have been reported **(AREMU et al. 2012a; GEMROTOVÁ et al. 2013)**. Inhibitors of cytokinin degradation regulate endogenous CK levels in plants as demonstrated in a recent study **(AREMU et al. 2012d)**. Being a CK antagonist, PI-55 compounds mimic the consequences of decreased cytokinin levels in plants which validate its competency in the inhibition of CK perception in plants **(SPÍCHAL et al. 2009)**. While smoke-technology has been widely utilized in traditional farming for decades **(KULKARNI et al. 2011)**, the recently isolated KAR_1 has shown potential in micropropagation as a PGR **(AREMU et al 2012a)**.

1.3. Therapeutic value of secondary metabolites in plants

Plants contain a variety of secondary metabolites which might be unique to a particular species or family. These secondary metabolites serve as taxonomic markers within species contributing to plant odour, taste and colour **(VERPOORTE et al. 2002)** as well as serving as sources of agrochemicals and biopesticides **(RAMACHANDRA RAO and RAVISHANKAR 2002)**. Besides being utilized as plant defence mechanisms, secondary metabolites have been exploited in the treatment of a wide variety of human ailments for centuries **(VAN WYK and WINK 2004)**. These natural products belong to diverse groups such as phenolics, terpenes, steroids and alkaloids **(BOURGAUD et al. 2001)**. Up to 40 000 terpenes, 20 000 phenolics and 5000 alkaloids have been identified **(CROTEAUS et al. 2000)**. Phenolics are distinguished by their involvement in lignin synthesis in all higher plants. Phenolic compounds include tannins (hydrolysable and condensed tannins) and flavonoids **(ROBARDS et al. 1999)**. Tannins and flavonoids possess diverse pharmacological activities such as immunomodulating effects, antimicrobial, anti-inflammatory, anti-diarrhoeal, antiviral, anti-tumor, antioxidant, antiallergic, free radical scavenging, vasodilatory and lipid peroxidation inhibition properties **(COOK and SAMMA 1996; OKUDA 2005; TOMCZYK and LATTÉ 2009)**. On the other hand, the distribution and synthesis of alkaloids is genus and species-specific **(BOURGAUD et al. 2001)**. Alkaloids have been implicated in a wide variety of activities including antibacterial, antimalarial **(YAMAMOTO et al. 1993; IWASA et al. 1998)**, anti-inflammatory **(DELLA LOGGIA et al. 1989)** anti-histaminic, anti-allergenic, anti-mutagenic angioprotective properties **(AMSCHLER et al. 1996)**. Even though

secondary metabolites are synthesized in low concentrations, their diversity and abundance in the plant kingdom contribute to their high importance. Consequently, it becomes pertinent to up-regulate their production in plants.

Micropropagation offers an effective tool to increase the production of secondary metabolites in target cells or tissues, this can be achieved by manipulating the chemical and physical conditions of the *in vitro* environment **(DiCOSMO and MISAWA 1995)**. As such, there is a rapid increase in the number of studies focusing on the enhancement of secondary metabolites in medicinal plant species globally **(MATKOWSKI 2008; COSTE et al. 2011; PAVARINI et al. 2012; AMOO and VAN STADEN 2013a)**. Most importantly, *in vitro* manipulation for the production of secondary metabolites remain a potential source which can guarantee a steady supply for pharmaceutical or nutraceutical industries **(VERPOORTE et al. 2002)**.

1.4. *Eucomis autumnalis* subspecies *autumnalis*

Eucomis (Hyacinthaceae) is a relatively small genus and widely distributed in several African countries including South Africa. *Eucomis autumnalis* subspecies *autumnalis* is widely exploited for its medicinal values and has been reported to possess anti-inflammatory properties **(TAYLOR and VAN STADEN 2001a; TAYLOR and VAN STADEN 2002a)**. Furthermore, the plant is in high demand in the horticultural industry due to its long lasting ‘eye-catching’ flowers resembling a pineapple. In an effort to conserve the species, micropropagation protocols have been documented **(TAYLOR**

and VAN STADEN 2001b). However, a thorough understanding of the different factors affecting growth and phytochemical content of the regenerants is insufficient.

1.4. Aims and objectives

The current study was aimed at better understanding the different factors essential for improving growth and secondary metabolite production in micropropagated *E*. *autumnalis* subspecies *autumnalis*.

The main objectives of the project were to evaluate:

- The effect of gelling agents (GA) and explant source (ES) on growth and secondary metabolite content in tissue culture regenerants;
- The effect of CKs [topolins in comparison with benzyladenine (BA)] in combination with auxin on growth and secondary metabolite content in tissue culture regenerants; and
- The effect of SW, KAR_1, PI-55 and INCYDE on growth and secondary metabolite content in tissue culture regenerants.

1.5. General overview of the thesis

Chapter 2 highlights the extensive use of the genus *Eucomis* in African Traditional medicine (ATM) and its pharmacological potential. It further critically reviews the different propagation techniques utilized in the conservation of the genus.

Chapter 3 describes the influence of GA, ES and their interaction with PGRs in the enhancement of growth and development as well as secondary metabolite production on *E*. *autumnalis* subspecies *autumnalis*.

Chapter 4 evaluates the influence of CKs and auxins on shoot proliferation. In addition, the recently identified CKs (*meta*-topolins) were compared to the widely used BA in terms of improved growth, secondary metabolite production and antioxidant activity. The study involve both *in vitro* culture stage and after 4 month of acclimatization in the greenhouse

Chapter 5 describes the potential of recently identified CK analogues and smoke compound (INCYDE, PI-55, KAR_1) as well as SW in enhancing growth, secondary metabolite content and antioxidant activity.

Chapter 6 presents a summary of the main findings of the study.

The section **'References'** provides a list of all the literature and materials cited in the thesis.

Appendix 1 represents the Murashige and Skoog (MS) basal medium protocol.

Appendix 2 shows the chemical structures of the auxin and cytokinins tested.

Chapter 2: Pharmacological potential and conservation prospects of the genus *Eucomis* (Hyacinthaceae) endemic to southern Africa

2.1. Introduction

The genus *Eucomis* is a member of the Hyacinthaceae (formerly included in the Liliaceae) comprising of 41 genera distributed in Europe, South America and Africa. In southern Africa, there are 27 genera and approximately 200 species of Hyacinthaceae found in the Cape Floristic Region, South Africa **(POOLEY 2005)**. Among the genera in southern Africa *Eucomis* is endemic to the region. The genus is relatively small consisting of 10 species **(POOLEY 2005)**. Although *Eucomis* species are generally summer blooming, *E*. *regia* is winter blooming. The species comprise of deciduous geophytes with long and narrow leaves topped with densely packed flowers **(COMPTON 1990)**. The characteristic leaf-like bracts at the tip of the flower spikes earned this genus its Greek name 'eukomes' which refers to 'beautiful headed' **(BRYAN 1989)**. *Eucomis* is commonly called the 'pineapple lily' because of the flower spikes that resemble pineapples **(PIENAAR 1984)**.

In African traditional medicine (ATM), *Eucomis* species are widely utilized against various ailments including respiratory, venereal diseases and rheumatism **(HUTCHINGS et al. 1996)**. Consequently, *Eucomis* species have been evaluated in both *in vitro* and *in vivo* bioassays for anti-inflammatory, antibacterial, antihistaminic and angioprotective potential **(RABE and VAN STADEN 1997; TAYLOR and VAN STADEN 2002a)**. The extensive biological activities of *Eucomis* species have been

mainly attributed to phytochemicals such as the homoisoflavanones commonly present in the plant. An in-depth review on the phytochemistry of the genus *Eucomis* is documented **(KOORBANALLY et al. 2006a)**. The extensive exploitation of *Eucomis* species in ATM together with its slow propagation rate has inevitably resulted in the decline of the majority of species, some of which are endangered/threatened **(RAIMONDO et al. 2009)**. The current **Chapter** focuses on the pharmacological characteristics of the genus *Eucomis*. A critical evaluation of available propagation protocols as a possible means of enhancing their conservation status is also discussed. Even though members of the genus *Eucomis* are widely utilized globally, the current **Chapter** is presented from a South African perspective.

2.2. Distribution and general morphology of *Eucomis* species

2.2.1. Distribution

Eucomis is widely exploited for its medicinal and horticultural value in southern African countries such as South Africa, Botswana, Lesotho, Swaziland, Zimbabwe and Malawi **(PIENAAR 1984; DU PLESSIS and DUNCAN 1989)**. In South Africa, *Eucomis* species are distributed across all nine provinces. In terms of species richness, the Eastern Cape with nine species is best represented, followed by KwaZulu-Natal with seven species while Northern Cape is the least endowed province with only one species **(Table 2.1)**. The distribution pattern of the genus *Eucomis* across the provinces varies depending on the species. While some species occur in two or more provinces, other species such as *E*. *humilis*, *E*. *montana*, *E*. *pallidiflora* subspecies *pole-evansii* and *E*. *zambesiaca* are found only in one province. This is a demonstration of their specificity to particular

climatic conditions in South Africa **(Table 2.1)**. Their growth and development depend on certain factors such as climatic regions and environmental conditions. For instance, *E*. *bicolor* is commonly found at the base of the Drakensberg cliffs along moist slopes ranging from 1800 - 2600 m while *E*. *humilis* is found on slopes that range from 2400 - 2900 m. *Eucomis autumnalis* is distributed in damp craters in grasslands that range from 2100 - 2400 m **(TRAUSELD 1969)**. The specificity of *Eucomis* species to certain environmental and climatic conditions are amongst the contributing factors that have resulted in the excessive decline and vulnerable status of the species in South Africa **(RAIMONDO et al. 2009)**.

Table 2.1: Distribution and ethnobotanical uses of members of the genus *Eucomis* found in South Africa.

Species	Province(s)	Plant part(s)	Traditional uses	References
E. autumnalis (Mill.) Chitt. (synonym *E. undulate*)	FS, KZN, EC, M, G	Leaves, bulbs, roots	Colic, flatulence, kidney and bladder problems, nausea, coughs syphilis, abdominal distension	**BISI-JOHNSON et al. (2010)**; **HUTCHINGS et al. (1996)**; **ROBERTS (1990)**; **WATT and BREYER-BRANDWIJK (1962)**
E. autumnalis (Mill.) Chitt. subspecies *amaryllidifolia* (Baker) Reyneke	L, FS, EC	NA	NA	NA
E. autumnalis (Mill.) Chitt. subspecies *autumnalis*	EC, L	Leaves, bulbs	Administered as enemas to treat lower backache, biliousness, urinary diseases, post-operative recovery, fevers and fractures	**HUTCHINGS et al. (1996)**
E. autumnalis (Mill.) Chitt. subspecies *clavata* (Baker) Reyneke	KZN, FS, M, G, L, NW	Bulbs	Administered as enemas to treat lower backache, biliousness, urinary diseases, post-operative recovery, fevers and fractures	**HUTCHINGS et al. (1996)**
E. bicolor Baker	EC, KZN	Bulbs	Colic and purgative	**WATT and BREYER-BRANDWIJK (1962)**; **HUTCHINGS et al. (1996)**
E. comosa (Houtt.)Wehrh. (synonym *E. punctate*)	EC, KZN	Bulbs, roots	For rheumatism, teething infants and purgative	**CUNNINGHAM (1988)**; **HUTCHINGS et al. (1996)**; **WATT and BREYER-BRANDWIJK (1962)**
E. comosa (Houtt.)Wehrh. variety *comosa*	WC, EC, KZN	Bulbs, roots	Rheumatism and teething in infants	**WATT and BREYER-BRANDWIJK (1962)**
E. comosa (Houtt.)Wehrh. variety *striata* (Don) Willd.	EC, L	NA	NA	NA
E. humilis Baker	KZN	NA	NA	NA
E. montana Compton	M	NA	NA	NA
E. pallidiflora Baker subspecies *pallidiflora*	EC, M	Bulbs	Mental diseases	**WATT and BREYER-BRANDWIJK (1962)**
E. pallidiflora subspecies *pole-evansii* (N.E.Br.) Reyneke ex J. C. Manning	M	Bulbs	Erectile dysfunction, tuberculosis, blood clotting, cough	**SEMENYA and POTGIETER (2013)**; **SEMENYA et al. (2013)**
E. regia L'Hér.	NC, WC	Bulbs, roots	Venereal diseases, diarrhoea, cough, biliousness and prevent premature childbirth	**WATT and BREYER-BRANDWIJK (1962)**
E. schijffii Reyneke	EC, KZN	Bulbs	Venereal diseases, diarrhoea, coughs and used as enema for biliousness, prevention of pre-mature birth, lower back pains	**WATT and BREYER-BRANDWIJK (1962)**
E. vandermerwei I. Verd.	M, L	NA	NA	NA
E. zambesiaca Baker	L	NA	NA	NA

NA- Not Available, EC- Eastern Cape, FS- Free State, G- Gauteng, KZN- KwaZulu-Natal, L- Limpopo, M- Mpumalanga, NC- Northern Cape, NW- North West, WC- Western Cape

2.2.2. General morphology

Eucomis species are geophytes with ovoid or globose shaped bulbs comprising of hard cortices **(BRYAN 1989; DE HERTOGH and LE NARD 1993)**. The bulb size ranges from 50 - 150 mm in diameter and has a perceptible horizontal striped base with brown to black scales **(Fig. 2.1A)**. The inner part of the bulb is yellow-white and turns black when exposed to air **(MANDER et al. 1995)**. The bulbs have branched perennial fleshy contractile roots with root hairs **(Fig. 2.1B)**. They are characterized by a rosette of smooth often shiny leaves that are lanceolate, elliptic or ovate and bend backwards **(Fig. 2.1C)**.

The stem ranges from 30 - 100 cm in height depending on the species. A straight cylindrical inflorescence with a pale lime-green flower raceme is located at the top of the flowering stem **(DE HERTOGH and LE NARD 1993)**. The flower colour varies from yellowish-green or white with margins varying from pale to dark purple. Flower colour turns green on maturity **(Fig. 2.1D)**. After pollination and fertilization, green or brown fruits appear containing dull blackish-brown seeds **(BRYAN 1989; DU PLESSIS and DUNCAN 1989)**.

The most common feature used for plant identification is flower morphology. However, the aforementioned method is difficult with the genus *Eucomis* because the flowers are morphologically similar. Therefore, features such as fragrance, plant size and leaf colour allows for better differentiation among species. Members of the genus emit

distinct floral scents. Sweet aroma species include *E*. *amaryllidifolia*, *E*. *autumnalis*, *E*. *comosa*, *E*. *grimshawii*, *E*. *pallidiflora* and *E*. *zambesiaca* while *E*. *bicolor*, *E*. *humilis*, *E*. *montana*, *E*. *regia*, *E*. *schijffii* and *E*. *vandermerwei* emit an unpleasant scent **(ZONNEVELD and DUNCAN 2010)**. The presence or absence of a purple colour at the leaf base or flower and the cylindrical shape of the scape are used to distinguish *Eucomis* species. Taken together, these characteristics become more difficult for identification and differentiation among closely-related species or subspecies. Therefore the use of genome size together with nuclear DNA content provides a better tool to distinguish species **(ZONNEVELD and DUNCAN 2010)**.

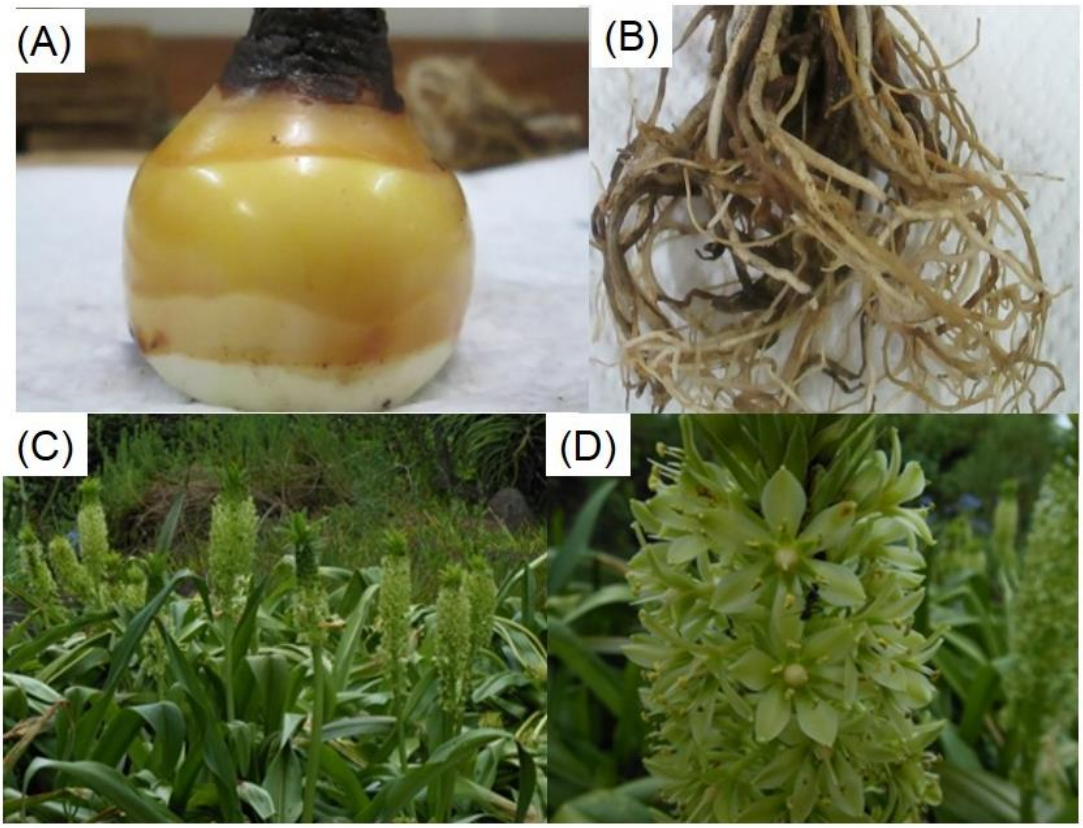


Fig. 2.1: Typical *Eucomis* morphology. A - bulb; B - roots; C - whole plants and D – inflorescence.

According to **REYNEKE and LIEBENBERG (1980)**, *Eucomis* species have 15 chromosomes which exist either in diploid (small species) or tetraploid (larger species) states. The diploid species (2*n*=2*x*=30) are *E*. *amaryllidifolia*, *E*. *bicolor*, *E*. *grimshawii*, *E*. *regia*, *E*. *schijffii*, *E*. *vandermerwei* and *E*. *zambesiaca* while the tetraploid species (2*n*=4*x*=60) include *E*. *autumnalis*, *E*. *comosa*, *E*. *humilis*, *E*. *montana* and *E*. *pallidiflora* **(ZONNEVELD and DUNCAN 2010)**. However, based on the variation in chromosome number of individual tetraploid species, **REYNEKE and LIEBENBERG (1980)** concluded that tetraploids are in fact allotetraploids. A detailed review focusing on the genome size of the different *Eucomis* species is available **(ZONNEVELD and DUNCAN 2010)**.

2.3. Horticultural potential of *Eucomis*

Since the middle 18th century, international interest in South African indigenous floriculture has intensified. The industry is known for its high economic value and potential job creation opportunities **(TAYLOR and VAN STADEN 2001b; REINTEN et al. 2011)**. Globally, the floriculture industry is worth an estimated US$9 billion annually **(BESTER et al. 2009)**. In 2011, with a turnover of approximately €18 million, the South Africa based Multiflora company in Johannesburg was ranked among the top 15 flora companies globally **(KRAS 2011)**. As an indication of their great potential especially for the international market, *Eucomis* species are in high demand in floriculture **(REINTEN et al. 2011)**.

Eucomis species are endowed with long lasting ‘eye-catching’ flowers, this attractive feature has inevitably intensified the demand for the species in the floriculture industry. Furthermore, longevity and wide adaptability to different environmental conditions are well-desired traits in *Eucomis* that have exacerbated the utilization of the genus as a cut flower. In addition to the floriculture industry, the sub-division known as the flower bulb industry facilitates the marketing of dry bulbs and potted plants in a controlled environment **(NIEDERWIESER et al. 1998)**. Thus far, *Eucomis* has shown great potential to be traded as dry bulbs and pot plants in the floriculture industry. Within the genus, small plants such as *E. zambesiaca* and *E*. *humilis* can be cultivated as pot plants whereas the larger species are better suited as garden plants.

2.4. Documented uses in African traditional medicine

As highlighted in **Table 2.1**, several *Eucomis* species are utilized as remedies against various ailments in ATM. In South Africa, plant materials are often prepared as decoctions, infusions and enemas. The Zulu, Tswana, Sotho and Xhosa people commonly use either water or milk for the preparing of these decoctions.

Evidence show that bulbs of *Eucomis* species are the most utilized plant part when compared to other organs such as roots, stems and leaves **(Table 2.1)**. However, bulbs and roots are occasionally combined as ingredients in infusions for alleviation of pain and fever **(HUTCHINGS et al. 1996)**. It has also been documented that the Zulu tribe use bulb infusions for the relief of biliousness, enhancing sexual prowess and cleansing of blood **(MANDER et al. 1995)**. Based on documented uses as summarized in **Table**

2.1, it is apparent that the majority of the *Eucomis* species are highly valued in ATM as demonstrated by their numerous uses e.g. treating kidney and bladder ailments as well as nausea and coughs. In folk medicine, it is believed that bulbs possess mysterious powers and they are used as protective charms **(WATT and BREYER-BRANDWIJK 1962)**.

Different plant species are combined and used together as a common practice in ATM. Along this line, *Eucomis* with *Crinum*, *Bowiea*, *Xanthoxylum* and *Becium* are combined and used as a form of treatment against cancer **(FENNELL and VAN STADEN 2001)**. The leaves are used as a poultice for sores and boils and are wrapped around the wrists to reduce fever. Apart from the excessive use of the plant in the treatment of human diseases, *Eucomis* species also serve as a remedy for animal ailments. The leaves and bulbs of *Eucomis* are combined with *Medicago sativa* or *Zea mays* leaves to treat gall sickness and other diseases in cattle **(ROBERTS 1990)**. Moreover, the plant is used for the treatment of venereal diseases in livestock **(WATT and BREYER-BRANDWIJK 1962; HUTCHINGS et al. 1996)**.

2.5. Phytochemistry and pharmacology of *Eucomis* species

The extensive traditional use of *Eucomis* species has led to several pharmacological properties being evaluated. The increasing number of ethnopharmacological studies has shown the potential substitution and supplementation of synthetic drugs with extracts and/or isolated compounds from medicinal plants **(RATES 2001; NEWMAN et**

al. 2003). The phytochemical diversity in higher plants accounts for their promising pharmacological potential.

2.5.1. Phytochemistry

Several classes of phytochemicals have been isolated from *Eucomis* species including homoisoflavanones, spiocyclic nortriterpenes, benzopyranones amongst others. Approximately 39 constituents commonly found in the Hyacinthoideae genera have been isolated from six *Eucomis* species **(POHL et al. 2000)**. However, eight species including *E*. *autumnalis* subspecies *amaryllidiflolia*, *E*. *autumnalis* subspecies *clavata*, *E*. *comosa* subspecies *striata*, *E*. *pallidiflora* subspecies *pallidiflora*, *E*. *regia*, *E*. *humilis*, *E*. *vandermerwei* and *E*. *zambesiaca* require more studies for possible isolation of novel compound(s). Recently, the phytochemical content of members of *Eucomis* has been extensively reviewed by **KOORBANALLY et al. (2006a and b)**.

The presence and wide diversity of flavonoids in *Eucomis* species has been associated with their pharmacological properties for example, anti-inflammatory activity **(HELLER and TAMM 1981)**. Often, pain and inflammation are common underlying symptoms in the majority of ailments treated with *Eucomis* species **(KOORBANALLY et al. 2005)**. The large number of isolated compounds from *Eucomis* plants is an indication of the value of the genus as a potential candidate for new possible drugs in the pharmaceutical industry for pain related ailments and bacterial/fungal infections.

2.5.2. Pharmacology

In an attempt to rationalise the wide usage and validate the efficacy of medicinal plants, researchers often evaluate bioactivities under laboratory conditions. *Eucomis* species have been mainly screened for bioactivities such as anti-inflammatory **(Table 2.2)** and antimicrobial **(Table 2.3)** properties. Based on the numerous benefits associated with *in vitro* test systems, such bioassays remain popular and a widely-used approach by researchers **(HOUGHTON et al. 2007)**. As evident in the current Chapter, the majority of documented studies were conducted using *in vitro* methods.

2.5.2.1. Anti-inflammatory screening

Inflammation processes involve the production of prostaglandins which are highly active pro-inflammatory mediators **(ZSCHOCKE et al. 2002)**. The biosynthesis of prostaglandin is regulated by cyclooxygenase (COX) enzymes **(JÄGER and VAN STADEN 2005)**. The enzymes occur in two major isoforms namely COX-1 and COX-2. While COX-1 contribute to the homeostasis of numerous physiological functions in different tissues, COX-2 is involved in several inflammatory reactions caused by inflammatory stimuli such as mitogens and cytokinesis **(KUJUBU et al. 1991; O'BANION et al. 1991)**. In an effort to alleviate inflammation, several plant extracts have been screened *in vitro* for prostaglandin synthesis inhibition. In ATM, *Eucomis* species are commonly utilized for inflammation and pain related ailments **(Table 2.1)**. As such, the majority of the pharmacological screenings have focused on their anti-inflammatory potential **(Table 2.2)**. Varying levels of anti-inflammatory activity have

been detected in several *Eucomis* species. Even though the majority of species exhibited high COX-1 or COX-2 enzyme inhibition, *E*. *autumnalis*, *E*. *autumnalis* subspecies *autumnalis*, *E*. *autumnalis* subspecies *amaryllidiflolia* and *E*. *humilis* were the most active. Among researchers, there are concerted efforts at discovering COX-2 preferential inhibitors as a result of the numerous side effects associated with COX-1 inhibitors **(WALLACE and CHIN 1997)**. Therefore, the selective inhibition of COX-2 by *E*. *autumnalis* subspecies *autumnalis* extract is worth pursuing for possible isolation of such desired bioactive compound(s).

In addition to the well-known effects of species-type on the anti-inflammatory activity, stringent studies aimed at the better understanding of other crucial factors affecting *Eucomis* species inhibition of COX have been conducted. In relation to extracting solvent, many of the evaluated *Eucomis* species exhibited high COX-1 and COX-2 activity when extracted with ethanol or 70% acetone as compared to water extracts **(Table 2.2)**. Generally water extracts are known to exhibit lower bioactivity when compared to non-polar extracts in various pharmacological studies. However, **JÄGER et al. (1996)** showed that extracting solvents had no significant effect on the anti-inflammatory activity of *E*. *autumnalis*. This was observed from the high activity of prostaglandin-synthesis inhibition in water (73%) and ethanol (90%) extracts of *E*. *autumnalis* at 0.5 mg/ml. Furthermore, the majority of the anti-inflammatory studies on the *Eucomis* genus have shown the potency of bulb extracts against both COX-1 and COX-2 enzymes **(Table 2.2)**. However, leaf extracts of species such as *E*. *bicolor* **(TAYLOR and VAN STADEN 2001a)** and *E*. *autumnalis* subspecies *autumnalis* **(TAYLOR and VAN STADEN 2002a)** showed higher enzyme inhibitory activity

compared to the bulb extracts. In addition, COX-2/COX-1 ratio for leaves, bulbs and root showed that leaf extracts (1.9) were more effective against COX-1 while bulbs and roots were effective against COX-2 enzyme (0.8 and 0.7 respectively). The high COX-1 activities depicted by leaf extracts of *E*. *autumnalis* subspecies *autumnalis* create an awareness of the efficacy in other plant parts besides the vulnerable bulbs. The preferential COX-2 inhibition by bulb and root extracts of *E*. *autumnalis* subspecies *autumnalis* is an indication of its pharmaceutical potential **(TAYLOR and VAN STADEN 2002a)**.

The increased demand for *Eucomis* plants in both formal and informal markets has exacerbated harvesting from wild populations. In order to prevent plant pathogens from attacking fresh plant materials, plants are dried, stored and sold later. Due to such practices, there is increasing concern on the pharmacological potency of dried medicinal plants as compared to fresh plant materials **(ELOFF 1999; STAFFORD et al. 2005; LAHER et al. 2013)**. Several authors have reported variation in anti-inflammatory activity among stored and fresh plant material **(FENNELL et al. 2004)**. **STAFFORD et al. (2005)** showed the effectiveness of both stored (90 days) and fresh *E*. *autumnalis* extracts with 100% inhibition. Furthermore, **TAYLOR and VAN STADEN (2002b)** reported high COX-1 activity (approximately 70%) from *E*. *autumnalis* subspecies *autumnalis* stored (dormant) in cold conditions (10 °C) compared to those maintained at 15 - 24 °C (55%).

Table 2.2: Examples of *in vitro* studies screening different *Eucomis* species for anti-inflammatory activity.

Species	Extracting solvent	Finding(s) and extract concentration	Indomethacin inhibition (concentration)	Reference(s)
E. autumnalis	Ethanol, water	High COX enzyme inhibition in bulb extracts from ethanol (90%) at 0.5 mg/ml and water (73%) at 0.5 mg/ml	66. 5% inhibition (0.5 µg)	**JÄGER et al. (1996)**
E. autumnalis	NR	Bulb extract showed 88% inhibition	NR	**GAIDAMASHVILI and VAN STADEN (2006)**
E. autumnalis	70% acetone, water	70% acetone extracts at 250 µg/ml had ≥ 75% enzyme inhibition except for smoke-water (COX-1), the control and light exposure treatment (COX-2)	64.2% inhibition (5µM)	**NDHLALA et al. (2012)**
E. autumnalis subspecies *amaryllidifolia*	Ethanol, water	COX-1 activity from ethanol at 250 µg/ml and water extract at 500 µg/ml was moderate (40 - 70%)	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. autumnalis subspecies *amaryllidifolia*	Ethanol	Ethanol bulb extract at 250 µg/ml exhibited high activity against COX-1 and COX-2 inhibitors (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2=200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. autumnalis subspecies *autumnalis*	Ethanol, water	IC_{50} values COX-1 from ethanol extracts at 250 µg/ml were evaluated. IC_{50} value for leaf extract was 15, for bulb extract was 72 and for root extract was 27 µg/ml	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. autumnalis subspecies *autumnalis*	Ethanol	Ethanol bulb extracts at 250 µg/ml exhibited high COX-1 and COX-2 activity (70 - 100%). IC_{50} ratio for COX-1 to COX-2 was 1.9 (leaf), 0.8 (bulb) and 0.7 (root)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. autumnalis subspecies *autumnalis*	Ethyl acetate, hexane	Bulb and root extracts at 250 µg/ml had higher inhibitory activity (> 90%) than leaf extract (65%) against COX-1	80% inhibition (20 µM)	**ZSCHOCKE et al. (2000)**
E. autumnalis subspecies *autumnalis*	Ethanol, water	Both ethanol extracts at 50 mg/ml of fresh and stored plant materials exhibited 100% COX-1 inhibition while water extracts showed ≤ 37% inhibition	64% inhibition (50 µg/ml)	**STAFFORD et al. (2005)**
E. autumnalis subspecies *clavata*	Ethanol, water	COX-1 activity from ethanol at 250 µg/ml and water extract at 500 µg/ml was moderate (40 - 70%)	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. autumnalis subspecies *clavata*	Ethanol	Ethanol bulb extract at 250 µg/ml exhibited high activity against COX-1 and COX-2 inhibitors (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. bicolor	Ethanol, water	COX-1 activity from leaf ethanol at 250 µg/ml and water extracts at 500 µg/ml, ethanol bulb extract was high (70 - 100%)	Activity NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. comosa	Ethanol	Ethanol bulb extract at 250 µg/ml exhibited high activity against COX-1 and COX-2 inhibitors (70-100%)	Inhibition NR (COX-1= 5 µM)	**TAYLOR and VAN STADEN (2002a)**

Species	Extracting solvent	Finding(s) and extract concentration	Indomethacin inhibition (concentration)	Reference(s)
			NR (COX-2= 200 µM)	
E. comosa subspecies *comosa*	Ethanol, water	COX-1 activity from ethanol at 250 µg/ml and water extract at 500 µg/ml was moderate (40 - 70%)	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. comosa subspecies *comosa*	Ethanol	Ethanol bulb and root extract at 250 µg/ml exhibited high activity against COX-1 inhibitors (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. comosa subspecies *striata*	Ethanol, water	COX-1 activity from ethanol 250 µg/ml and water extract 500 µg/ml was moderate (40 - 70%)	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. comosa subspecies *striata*	Ethanol	Ethanol bulb extract 250 µg/ml exhibited high activity against COX-1 and COX-2 inhibitors (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. humilis	Ethanol, water	COX-1 and COX-2 activity from ethanol 250 µg/ml and water bulb extracts 500 µg/ml was high (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**
E. zambesiaca	Ethanol, water	COX-1 activity from ethanol at 250 µg/ml and water extract 500 µg/ml was moderate (40 - 70%)	Inhibition NR (5 µM)	**TAYLOR and VAN STADEN (2001a)**
E. zambesiaca	Ethanol	Ethanol bulb and root extract at 250 µg/ml exhibited high COX-1 activity (70 - 100%)	Inhibition NR (COX-1= 5 µM) NR (COX-2= 200 µM)	**TAYLOR and VAN STADEN (2002a)**

NR- Not reported; COX = cyclooxygenase

2.5.2.2. Antimicrobial screening

The increase in drug resistance and side effects with the frequently used medications (mainly antibiotics) are well-known. Consequently, enormous efforts have been geared towards the screening of medicinal plants as a potential source of novel leads in the treatments of microbial infections **(RATES 2001)**. Different plant parts and extracting solvents have been used in the *in vitro* and *in vivo* screening of several *Eucomis* species. The effect of *Eucomis* extracts on diverse microbes such as *Staphylococcus aureus*, *S*. *epidermis*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Escherichia coli*, *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella pinodes*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Vericillium dahlia*, *Brotryosphaeria dothidea*, *Pythium ultimum* and *Candida albicans* have been investigated **(Table 2.3)**. *Eucomis* extracts inhibited only a few bacterial strains such as *B*. *subtilis*, *E*. *coli* and *S*. *aureus*. In a recent study **(BISI-JOHNSON et al. 2011)**, ethyl acetate extracts of *E*. *autumnalis* showed remarkable minimum inhibitory concentration (MIC) activity (0.27 mg/ml) against *E*. *coli*. Furthermore, **NDHLALA et al. (2012)** showed the activity of *E*. *autumnalis* against *B*. *subtilis* and *S*. *aureus* with an MIC value of 0.78 mg/ml. Generally, compounds are often isolated from crude extracts with antimicrobial potential. Five compounds isolated from *E*. *comosa* and *E*. *schijffii* showed significant MIC values (0.52 and 0.24 mM) against *S*. *aureus* **DU TOIT et al. (2007)**. Some of the compounds isolated by the authors include (1) = 1, 7-hydroxy-5-methoxy-3-(4′-hydroxybenzyl)-4-chromanone; (2) = 5,7-dihydroxy-8-methoxy-3 (4′ hydroxybenzyl)-4-chromanone (3,9-dihydropunctatin); (8)

= scillascillin and (9) = 23S-17α,23-epoxy-3β,28,29-trihydroxy-27-norlanost-8-en-24-one.

When *Eucomis* was tested against *C*. *albicans* the extracts showed a MIC value of ≥ 1.56 µg/ml **(MOTSEI et al. 2003; NDHLALA et al. 2012)**. In other studies, *E*. *autumnalis* extracts were not effective against *C*. *albicans* strain **(MOHLAKOANA 2010)**. Although *Eucomis* species were not very effective against *C*. *albicans*, the plants may be effective against other fungal strains. Therefore further research needs to be conducted on the antifungal properties of *Eucomis* species using other fungal strains, which if effective, can then be further tested *in vivo*. As a potential biocontrol agent, *E*. *autumnalis* subspecies *clavata* exhibited significantly high antifungal activity against seven plant pathogens **(EKSTEEN et al. 2001)**. In addition, *E*. *autumnalis* subspecies *clavata* extracts had ≥73% inhibition against six plant pathogens in a field trial **(PRETORIUS et al. 2002)**. The potential of *E*. *autumnalis* subspecies *clavata* against plant pathogens is noteworthy as it could provide an affordable and accessible means of controlling plant pathogens in agriculture.

Table 2.3: Examples of studies screening different South African *Eucomis* species for antimicrobial activity.

Species	Extracting solvents	Test system and organism(s)	Finding(s)	Positive control activity	References
E. autumnalis	Methanol, water	*In vitro* - five bacterial strains	Methanol bulb extract at 1 mg/ml extract exhibited a ratio of 0.13 zone of inhibition against *Bacillus subtilis* when compared to the positive control	10 µl of neomycin (200-500 µg/ml) was used in each petri-dish in the agar diffusion assay	**RABE and VAN STADEN (1997)**
E. autumnalis	Ethanol, ethyl acetate, hexane, water	*In vitro* - two clinical and one standard *Candida albicans*	Bulb extracts had minimum inhibitory concentration (MIC) > 8.35 mg/ml	Amphotericin B MIC activity 1.56 µg/ml	**MOTSEI et al. (2003)**
E. autumnalis	Methanol, ethyl acetate, butanol, water	*In vitro* - two *Escherichia coli* strains	Ethyl acetate extract had an MIC of 0.27 mg/ml	Kanamycin with an MIC of 0.195 mg/ml	**BISI-JOHNSON et al. (2011)**
E. autumnalis	70% acetone, water	*In vitro* - four bacterial strains and *Candida albicans*	Acetone bulb extracts had an MIC of 0. 78 mg/ml against *B. subtilis* and *S. aureus*	Neomycin MIC activity *B. subtilis* 1.531×10^{-3} *Staphylococcus aureus* 6.125×10^{-3}	**NDHLALA et al. (2012)**
E. autumnalis	Acetone, methanol, water	*In vitro* - 94 microbial strains	The leaf extracts were not active at the highest tested concentration (20 mg/ml)	27 different antibiotic were used for the assay	**MOHLAKOANA (2010)**
E. autumnalis subspecies *clavata*	Methanol	*In vitro* - seven plant fungal strains	Whole plant extract at 100 mg/ml had high inhibition against *Brotryosphaeria dothidea* (85%) and *Pythium ultimum* fungicide (95.4%)	Carbendazim/difenoconazole (Eria@ - 187.5 g/l EC) About 100% inhibition against three of the strains (1 µg/ml)	**EKSTEEN et al. (2001)**
E. autumnalis subspecies *clavata*	Methanol	*In vitro* - eight plant fungal strains	Bulb extracts at 1 mg/ml had a significant (≥73%) growth inhibition against six of tested fungal strains	Carbendazim/difenoconazole (Eria@ - 187.5 g/l EC) About 100% inhibition against two of the strains (1 µg/ml)	**PRETORIUS et al. (2002)**
E. autumnalis subspecies *clavata*	Methanol	*In vivo* - one plant fungal strain	Extract concentration of 1 mg/ml prevented spore infection (*Mycosphaerella pinodes*) in pea plant	Carbendazim/difenoconazole (Eria@ - 187.5 g/l EC) 1 µg/ml prevented spore infection (1 µg/ml)	**PRETORIUS et al. (2002)**
E. comosa	Methanol	*In vitro* - one bacterial strain	Compounds 1 and 2 had high inhibitory activity against *S.*	Neomycin MIC activity 0.0025 mM	**DU TOIT et al. (2007)**

Species	Extracting solvents	Test system and organism(s)	Finding(s)	Positive control activity	References
			aureus with MIC value ≤ 0.52 mM. Compound 9 (0.98 mM) had better inhibitory activity compared to compound 8 (4.15 mM). Compound 8 showed bacteriostatic activity (2.07 mM)		

MIC - minimum inhibitory concentration, NR - Not reported, Compounds (1) = 1, 7-hydroxy-5-methoxy-3-(4′-hydroxybenzyl)-4-chromanone; Compounds (2) = 5,7-dihydroxy-8-methoxy-3 (4′ hydroxybenzyl)-4-chromanone (3,9-dihydropunctatin) Compounds (8) = scillascillin; (9) = 23S-17α,23-epoxy-3β,28,29-trihydroxy-27-norlanost-8-en-24-one

2.5.2.3. Other pharmacological properties

Besides the aforementioned pharmacological properties, *Eucomis* has been tested for cytotoxicity, phytotoxicity, anticancer and anti-plasmodial activities **(Table 2.4)**. *Eucomis* had noteworthy antitumor and cytotoxicity activity. *Eucomis autumnalis* was evaluated for anticancer cell activity and the methanol extracts showed good activity (IC_{50} 7.8 µg/ml) against the human hepatoma cell line (Huh-7) compared to the positive control with an IC_{50} of 9.8 µg/ml **(BISI-JOHNSON et al. 2011)**. When *Eucomis* was screened for antitumor activity **(MIMAKI et al. 1994)**, eucosterol glycoside isolated from *E*. *bicolor* showed 44% inhibition of 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated ^{32}P incorporation into phospholipids of HeLa against tumor-promoters. *In vivo* tests are essential for further validation of *Eucomis* extracts as anti-cancer agents. This may be of great value in the search for anticancer drugs with potential lesser side effects as compared to other synthetic drug treatments. Furthermore, when *E*. *autumnalis* were evaluated for phytotoxicity the bulb extract (up to 2 mg/ml) were not toxic to pea leaves **(PRETORIUS et al. 2002)** and 1 mg/ml inhibited spore germination.

Table 2.4: Additional *in vitro* activities of South African *Eucomis* species.

Species	Extracting solution	Bioactivity	Report on the activity	Positive control activity (concentration)	References
E. autumnalis	Dichloromethane, Dichloromethane: Methanol (1:1) and water	Antiplasmodial activity	The bulb extracts from dichloromethane extract (70 µg/ml), Dichloromethane:Methanol (9.5 µg/ml) and water (100 µg/ml) IC_{50} value against *Plasmodium falciparum*	Chloroquine diphosphate (NR)	**CLARKSON et al. (2004)**
E. autumnalis	Methanol	Cytotoxicity activity	The methanol extracts were cytotoxic with an IC_{50} value of 7.8 µg/ml	Berberine IC_{50} = 9.8 µg/ml	**BISI-JOHNSON et al. (2011)**
E. autumnalis subspecies *clavata*	Methanol	Phytotoxicity	Up to 2mg/ml of bulb extract showed no phytotoxic effect	Carbendazim/difenoconazole (Eria$^{@}$ - 187.5 g/l EC) NR (1µg/ml)	**PRETORIUS et al. (2002)**

NR- Not Reported

2.6. Safety and toxicity of *Eucomis*

The issues of quality control and safety of conventional drugs are important aspects for the pharmaceutical industry. In ATM however, there is limited information on the safety of plant extracts or herbal products because of the presumed safety of natural products. Even though the *Eucomis* genus is extensively utilized in traditional medicine it has been implicated in human poisoning and death in sheep **(WATT and BREYER-BRANDWIJK 1962)**. According to **HUTCHINGS et al. (1996)** abdominal pain, diarrhoea and renal failure are some of the symptoms caused by *Eucomis* poisoning in humans. Poisoning may be due to the haemolytic toxin contained in the plants **(MANDER et al. 1995)**. Although the plant is a member of the Hyacinthaceae family, cardiac glycosides which are widely distributed in the family have not been detected in *Eucomis* species **(WATT and BREYER-BRANDWIJK 1962)**. As recently reviewed by **KOORBANALLY et al. (2006a)**, there is increasing evidence on the toxicity of crude extracts and isolated compounds from a number of *Eucomis* species. Nevertheless, the limited (if any) information on safety evaluation remain worrisome. Therefore, it would be pertinent to subject the various *Eucomis* species especially the ones demonstrating potent bioactivity to a sequence of toxicological and mutagenic (both *in vitro* and *in vivo*) evaluations. The effect of mode of administration, dosage and age as well as gender on incidences of toxicity requires investigation. Such valuable information will be vital in ATM as well as from scientific and commercialization perspectives.

2.7. Conservation status

Although legislation protecting medicinal plants including *Eucomis* has been established **(MANDER et al. 1995)**, harvesting of plants from their natural habitats continue unabated **(TAYLOR and VAN STADEN 2001b)**. Amongst the *Eucomis* species, *E*. *autumnalis* is the most widely used species **(WATT and BREYER-BRANDWIJK 1962; HUTCHINGS 1989; ROBERTS 1990; HUTCHINGS et al. 1996)**. As indicated by street traders, *E*. *autumnalis* and *E*. *bicolor* are amongst the most popular and widely traded species in Durban, South Africa **(CUNNINGHAM 1988; MANDER 1998)**. According to **CUNNINGHAM (1990)**, *E*. *autumnalis* is the second most widely traded species in KwaZulu-Natal, South Africa. As a result, *E*. *autumnalis* populations have been reported as declining **(MANDER 1998)**. Recently, *E*. *autumnalis* was listed as endangered by the International Union for the Conservation of Nature (IUCN) **(VICTOR 2000)**. Increased harvesting of *E*. *autumnalis* has significantly contributed to the shortage of the species in informal medicinal markets in South Africa **(GOVENDER et al. 2001)**. **Table 2.5** provides a summary of the conservational status of members of the genus *Eucomis*. Apart from the medicinal uses of *Eucomis*, potential of the species as an ornamental/horticultural plant due to their 'eye-catching' flowers has exerted more strain on wild populations. Therefore, the propagation of *Eucomis* species especially the widely utilized *E*. *autumnalis* and the vulnerable *E*. *vandermerwei* remains of outmost importance for the conservation of members of the genus.

2.7.1. Conventional propagation

Eucomis species can be propagated conventionally as offsets and by seeds. However, the propagation process is very slow **(VERDOORN 1973)**. On average, they reach maturity within approximately 3 - 4 years, and a bulb diameter of around 12 cm is required for floral initiation. Generally, the plant requires sunny or partially shaded areas/habitats for improved growth. Seed propagation is an effective method in conserving *Eucomis* species but knowledge of its seed biology is very limited. A germination rate of approximately 65% had been reported **(DIEDERICHS et al. 2002)**.

2.7.1.1. Response of *Eucomis* to conventional propagation

In a systematic approach to enhance conservation of *Eucomis*, stringent experiments on factors that influence its seed biology were conducted **(KULKARNI et al. 2006)**. The study showed that *E*. *autumnalis* subspecies *autumnalis* seed germination was inhibited by light, which implies the importance of dark conditions for seed germination. Furthermore, the authors discovered that cold-stratification (5 °C) for 45 days inactivates the inhibitory effect caused by light. Additives such as smoke-water (SW) and its isolated compound (butenolide = 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, otherwise known as karrikinolide = KAR_1) at varying concentrations were found to stimulate germination of *E*. *autumnalis* subspecies *autumnalis* **(KULKARNI et al. 2006)**. Enhanced germination refers to a new family of plant growth regulator‟s (PGR‟s) identified in smoke from burning plant material **(Dixon et al., 2009)**.

NDHLALA et al. (2012) reported on the essential and optimum environmental conditions required for *E*. *autumnalis* seedlings. For instance, the level of light exposure was vital for *E*. *autumnalis* seedling growth. Upon testing three light exposure (50, 75 and 100%), 50% light proved to be optimal resulting in superior shoot and root growth as well as the highest fresh weight in seedlings. In terms of the temperature, 25 °C and alternating 30/15 °C were the most preferred for better seedling growth. Application of SW (1:250 v/v) dilution significantly enhanced the seedling growth. **VAN LEEUWEN and VAN DER WEIJDEN (1997)** showed that *E*. *comosa* responded better when kept for 12 weeks in vermiculite at 17, 20 or 23 °C. However, there was no significant difference with *E*. *bicolor* when placed in vermiculite for 12 weeks at different temperatures. Furthermore, **KNIPPELS (2000)** showed that a period of 13 weeks in vermiculite was best for bulb growth and prevented premature death of bulbs. Moreover, temperatures ranging from 20 - 22 °C were suitable for better bulb growth especially *E*. *bicolor*. Despite the increasing number of studies focusing on the conventional propagation, it is evident that modern approaches such as micropropagation are necessary in order to alleviate the increasing strains on the wild population.

Table 2.5: Conservation status and micropropagation protocols for different *Eucomis* species.

Species	Conservation status	Explants used	Type of plant growth regulator(s)	Results/observations	References
E. autumnalis	Declining	Bulb twin-scale, bulb-scale, shoots, leaf	BA, NAA, NAA:BA, IAA, IBA	BA, BA and NAA showed significant shoot regeneration	**TAYLOR and VAN STADEN (2001b)**; **AULT (1995)**
E. autumnalis subspecies *amaryllidifolia*	Not evaluated	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA) with no shoots initiated from 1:2 mg/l media. Root initiation was achieved from 1 mg/l NAA	**TAYLOR and VAN STADEN (2001b)**
E. autumnalis subspecies *autumnalis*	Not evaluated	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation obtained from 1:1 mg/l (NAA:BA). Root initiation was achieved from 1 mg/l NAA	**TAYLOR and VAN STADEN (2001b)**
E. autumnalis subspecies *clavata*	Not evaluated	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA) with no shoots initiated from 1:2 mg/l. Root initiation was achieved from 1 mg/l NAA	**TAYLOR and VAN STADEN (2001b)**
E. bicolor	Near threatened	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:2 mg/l (NAA:BA) Root initiation was achieved from 1 mg/l IBA	**TAYLOR and VAN STADEN (2001b)**
E. comosa	Declining	Bulb twin-scale, bulb-scale, root, leaf	BA, NAA	Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA). BA and NAA had significant shoot regeneration	**AULT (1995)**; **TAYLOR and VAN STADEN (2001b)**
E. comosa subspecies *comosa*	Not evaluated	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:2 mg/l (NAA:BA). Root initiation was achieved from 1 mg/l IBA	**TAYLOR and VAN STADEN (2001b)**
E. comosa subspecies *striata*	Not evaluated	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA). Root initiation was achieved from 1 mg/l NAA	**TAYLOR and VAN STADEN (2001b)**
E. humilis	Least concern	Bulb, root, leaf	NAA:BA, IAA, IBA	Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA). Root initiation was achieved from 1 mg/l IBA	**TAYLOR and VAN STADEN (2001b)**
E. montana	Declining	NR	NR	NR	NR
E. pallidiflora subspecies *pallidiflora*	Least concern	NR	NR	NR	NR
E. pallidiflora	Near	NR	NR	NR	NR

Species	Conservation status	Explants used	Type of plant growth regulator(s)	Results/observations	References
subspecies *pole-evansii*	threatened				
E. *regia*	Least concern	NR	NR	NR	NR
E. *schijffii*	Least concern	NR	NR	NR	NR
E. *vandermerwei*	Vulnerable	Shoots, leaf	BA, IAA	Successful shoot initiation was obtain from explants propagated at 1 to 2 mg/l of BA and 1 mg/l IAA	**McCARTAN et al. (1999)**
E. *zambesiaca*	Least concern	Bulb twin-scale, bulb-scale, root, leaf	BA, NAA, NAA:BA, IAA, IBA, PBZ, 2,4-D, BA, iP, Zeatin, *m*T, GA_3, GA_{4+7}, ABA, MeJA, PAA	Significant shoot regeneration was obtained from NAA at 5,4 µM Induction of bulblets was obtained from 4.90 µM IBA Optimum shoot initiation was obtained from 1:1 mg/l (NAA:BA). Root initiation was achieved from 1 mg/l IAA	**AULT (1995)**; **CHEESMAN et al. (2010)**; **TAYLOR and VAN STADEN (2001b)**

NR- Not Reported; Conservation status according to **RAIMONDO et al. (2009)**
2,4-D = 2,4 – Dichlorophenoxy acetic acid; iP = N^6-Isopentenyladenine; BA = Benzyladenine; GA_3 = Gibberellic acid; GA_{4+7} = GA_4 and GA_7 gibberellin mixture; IAA = Indole acetic acid; IBA = Indole butyric acid; MeJa = Methyl jasmonate; *m*T = *meta*-Topolin; NAA = α-Naphthalene acetic acid; PAA = Phenylacetic acid; PBZ = Paclobutrazol

2.7.2. Micropropagation of *Eucomis*

Although efforts geared towards improving conventional propagation is commendable, the slow growth of *Eucomis* species which may take as long as 3 - 4 years for bulb maturation remains a major concern. Inevitably, the application of valuable techniques such as micropropagation (known for its numerous benefits) has been embraced. The technique is useful for the conservation of species as it increases the production turn-over rate and reduces the growth duration significantly. The success of hybridization in *Eucomis* flowers has been partly attributed to the fact that the hybrids can be further micropropagated and remain true-to-type.

As an evident of the success and increasing application of micropropagation for the genus *Eucomis*, **Table 2.5** shows members of the genus that have been micropropagated. For instance, the micropropagation of *E*. *autumnalis* and *E*. *zambesiaca* using twin-scales has been successfully conducted **(AULT 1995)**. **McCARTAN and VAN STADEN (1995)** focused on the tissue culture of *E*. *pole-evansii* with the use of seedling explants while **McCARTAN et al. (1999)** devised a protocol for *E*. *vandermerwei*, one of the most vulnerable species within the genus. Micropropagation of 11 *Eucomis* species was conducted by **TAYLOR and VAN STADEN (2001b)**. An improved micropropagation protocol for *E*. *zambesiaca* was described by **CHEESMAN et al. (2010)**. No doubt, these protocols are valuable for the mass propagation of *Eucomis* species. Therefore, it will be pertinent to provide protocols for the other *Eucomis* species which have not received much attention. Using

the new group of cytokinin (CK) (topolins) which have been demonstrated to be valuable in micropropagation **(AREMU et al. 2012b)**, efforts aimed at improving shoot proliferation in *E*. *autumnalis* subspecies *autumnalis* remain important and forms the basis of the subsequent **Chapters**.

2.7.2.1. Effect of plant growth regulators on the micropropagation of *Eucomis*

Plant growth regulators (PGRs) are involved in the enhancement of cell division. For instance, addition of exogenous CK stimulate cell division thereby enhancing shoot proliferation **(BAYLISS 1985)**. In plants, CKs are involved in numerous developmental stages including seed germination, de-etiolation, chloroplast differentiation, apical dominance, flower and fruit development **(HABERER and KIEBER 2002)**. On the other hand, auxins play a crucial role in cell enlargement, elongation, root initiation, stem growth and cell division **(GASPAR et al. 1996)**.

Micropropagation of *Eucomis* species showed that combination of CKs and auxins significantly improve shoot regeneration. According to **AULT (1995)**, the combination of benzyladenine (BA) and naphthalene (NAA) resulted in the optimum number (2.8) of shoots per bulb explant for *E*. *zambesiaca* and *E*. *comosa*. When 11 of the *Eucomis* species were evaluated **(TAYLOR and VAN STADEN 2001b)**, optimum shoot initiation was obtained from a BA:NAA (1:1) combination. For leaf explants, 8 shoots were regenerated per explant with 2 - 3 shoots regenerated per bulb explant. The shoot explants regeneration from bulb explants show a similar trend **(AULT 1995; TAYLOR and VAN STADEN 2001b)**. Although auxins function in cell elongation and root

formation, indole butyric acid (IBA) treatment induced optimum bulblets on *E*. *zambesiaca* **(CHEESMAN et al. 2010)**. According to **GASPAR et al. (1996)**, addition of IBA to the media causes the auxin to be broken down and further metabolized in plant tissues. Further studies showed that IBA resulted in optimum shoot initiation and growth responses in bulblets when compared to indole acetic acid (IAA) in *Hyacinthus orientalis* **(YI et al. 2002)**.

2.8. Conclusions

The continuous exploitation of the genus in southern Africa and especially in South Africa is an indication of its pharmacological potential. In view of the potential of the genus in anti-inflammatory therapy, the high COX inhibitory activity of crude extracts of active species such as *E*. *autumnalis* subspecies *autumnalis* and *E*. *bicolor in vitro* should be further investigated through *in vivo* bioassays. In order to achieve any pharmaceutical potential, it will be necessary to eliminate false positive results by removal of compounds such as polyphenols, saponins and fatty acids in plant extracts which are known to affect enzyme-based bioassays. In terms of the antimicrobial potential, the genus *Eucomis* is reported to be effective against *B*. *subtilis*, *E*. *coli* and *S*. *aureus* as well as several plant fungal strains **(Table 2.3)**. In fact, crude extracts of *Eucomis* species were more potent than the positive control against plant pathogens such as *S*. *rolfsii*, *R*. *solani* and *P*. *ultimum* **(Table 2.3)**. Hence, *Eucomis* species can be potentially useful in the agricultural sector as a fungicide or biocontrol agent. Based on the inadequate evidence on the general safety of members of the genus, the need for detailed toxicological and mutagenic evaluation is recommended. From a

conservational perspective, renewed efforts aimed at cultivation and application of micropropagation techniques will definitely help alleviate the declining status of many of these heavily harvested species, particularly *E*. *autumnalis*. Consequently, more studies geared towards understanding the basic requirements of improving their cultivation and micropropagation processes are encouraged.

Chapter 3: Influence of gelling agents, explant source and plant growth regulators in micropropagated *Eucomis autumnalis* subspecies *autumnalis*

3.1. Introduction

Globally, the increasing population, anthropogenic activities, and deteriorating natural ecosystems have caused several plant species (especially medicinal plants) to become threatened and even extinct **(SHARMA et al. 2010)**. As one of the most common and widely used medicinal plants in African Traditional Medicine (ATM), anecdotal evidence of the uses and pharmacological efficacy of *Eucomis* species are well-documented **(Chapter 2)**. In an attempt to meet and sustain the increasing demand for medicinal species including *E*. *autumnalis* subspecies *autumnalis*, the application of micropropagation has become an accepted viable option for their conservation. The technique allows for mass propagation and genetic improvement as well as the enhancement of secondary metabolite levels in several plant species **(TRIPATHI and TRIPATHI 2003)**. In addition, the biosynthetic pathways of desired phytochemicals can be manipulated to increase the level of these chemicals which are easily extractable from the *in vitro* regenerants **(DiCOSMO and MISAWA 1995)**. The quantity and quality of phytochemicals in micropropagated medicinal plants remain crucial especially in terms of their pharmacological potential and general acceptability **(DÖRNENBURG and KNORR 1995)**. Despite the numerous advantages associated with micropropagation, several factors such as gelling agents, explant source, type and concentration of plant growth regulators (PGRs) are known to affect the overall success of the technique

(GEORGE 1993). As such, it often becomes necessary to manipulate these factors in order to optimize micropropagation protocols.

Agar and gellan gum (Gelrite^{TM}) are natural polysaccharides with the ability to gel at room temperature and remain the most popular gelling agents used in micropropagation. Agar is a neutral linear, molecule free of sulphates with alternative chain units of β-1,3-linked-D-galactose and α-1,4-linked 3,6-anhydro-L-galactose. The gel is a derivative of red-purple seaweeds also known as agarophytes **(MARIHNO-SORIANO and BOURRET 2003)** and has remained the most widely utilized solidifying agent in tissue culture media for years **(PUCHOOA et al. 1999)**. The extensive use of agar is attributed to its high gel clarity, stability and ability to prevent plant enzyme digestion. Nevertheless, several reports on its adverse side effects have been documented **(DEBERGH 1983; ARTHUR et al. 2004)**. It has been postulated that when agar chelates or adsorb nutrient ions, explant growth is retarded due to the unavailability of the essential elements in the media **(DEBERGH 1983; BORNMAN and VOGELMAN 1984; CONNER and MEREDITH 1984)**,

In contrast, gelrite is a linear polysaccharide composed of two residues of D-glucose and one residue of D-glucuronic acid and L-rhamnose. Gelrite readily gels in the presence of monovalent or divalent cations. It is considered as a more economical alternative to agar because approximately half the amount of gelrite is required to attain the equivalent gelling strength as agar **(PIERIK 1987)**. As a product of *Pseudomonas alodea*, it is characterized by consistent high quality and purity. Gelrite produces a firm and very clear gel in the absence of contaminants **(PIERIK 1987)**. However, the use of

gelrite has often been associated with the occurrence of hyperhydric shoots in several species **(FRANCK et al. 2004; ROJAS-MARTÍNEZ et al. 2010)**.

TAYLOR and VAN STADEN (2001b) have reported the importance of the micropropagation protocol as well as the role of factors affecting regeneration in *E*. *autumnalis* subspecies *autumnalis*. For instance, the influence of explants and PGRs on the species has been investigated. Nevertheless, the role of other intricate factors such as gelling agent (in the presence of different PGR combinations and explant source) known to contribute significantly to the quality and quantity of regenerants have received less attention. The current **Chapter** evaluated the effect of two gelling agents on shoot proliferation and secondary metabolite production in micropropagated *E*. *autumnalis* subspecies *autumnalis*. In addition, the response of *in vitro* regenerants to individual gelling agents in the presence of different PGR combinations and explant source (leaf or bulb) used for the initiation stage was investigated.

3.2. Materials and methods

3.2.1. Sources of plant growth regulators

Benzyladenine (BA) and α-naphthalene acetic acid (NAA) were purchased from Sigma-Aldrich (Steinheim, Germany). *Meta*-topolin (*m*T) was prepared as previously described by **DOLEŽAL et al. (2006)**.

3.2.2. Explant source, decontamination regime and culture initiation

Explants were obtained from stock plants of *E*. *autumnalis* subspecies *autumnalis* maintained at the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg, South Africa. After identification by Dr C. Potgieter a voucher specimen (Masondo 2) was prepared and deposited in the Bews Herbarium of the UKZN, Pietermaritzburg, South Africa.

Leaves and bulbs **(Fig. 3.1)** were excised from the stock plants and decontaminated according to a procedure described by **TAYLOR and VAN STADEN (2001b)**. Briefly, leaves were surface decontaminated using 70% ethanol for 5 min followed by 0.2% Benlate® (Du Pont de Nemour Int., South Africa) with a few drops of Tween 20 (polyoxyethylene sorbitan monolaurate, Saarchem, Krugersdorp, South Africa) for 10 min then sterilized in 1.75% sodium hypochlorite for 20 min. The explants were subsequently rinsed three times with sterile distilled water. On the other hand, bulbs were immersed in sterile distilled water for 30 min and decontaminated with 100% ethanol for 60 s followed by 0.2% Benlate® with a few drops of Tween 20 for 5 min and 3% sodium hypochlorite for 20 min. The plant materials were rinsed three times with sterile distilled water to remove all traces of sterilants.

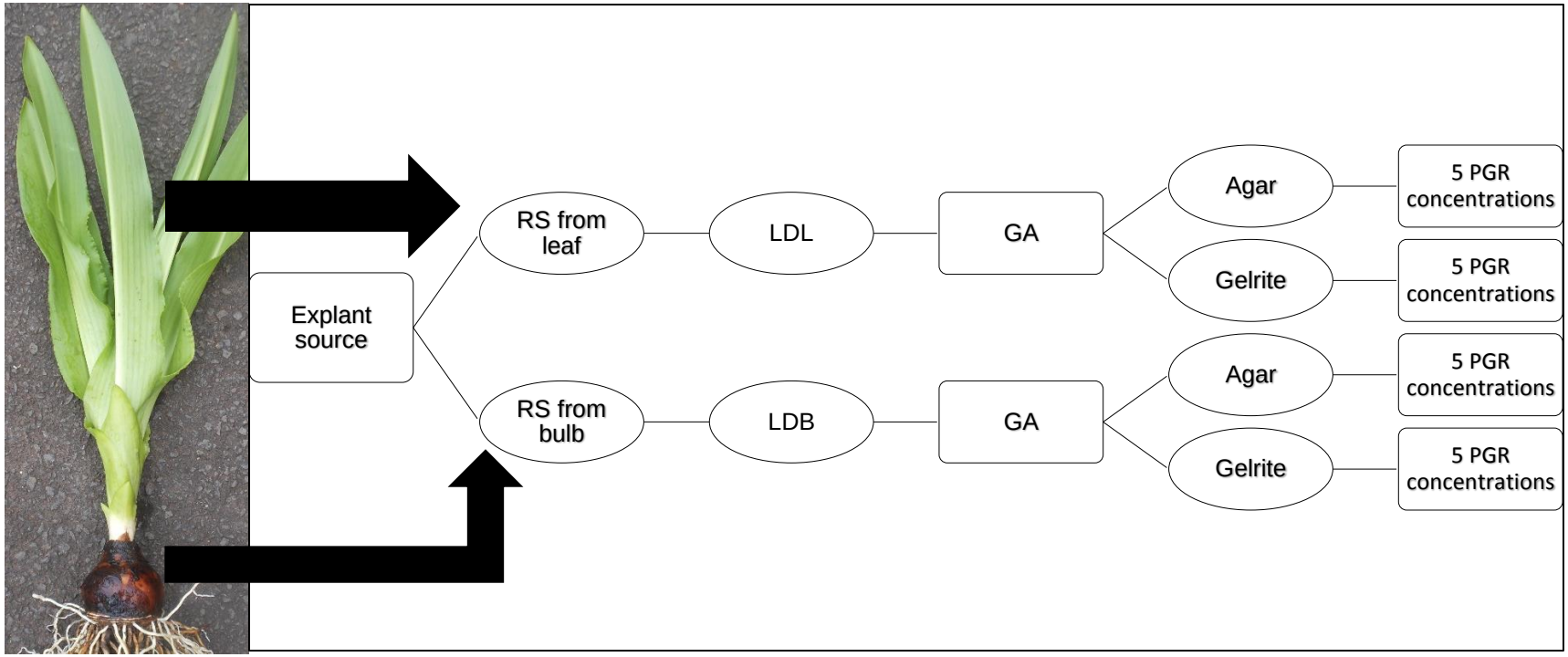


Fig. 3.1: Illustration on how the explants were obtained and experimental design indicating the three factors (rectangular shape) evaluated in the current study. RS = regenerated shoots; LDL = leaf explant derived from primary leaf regenerants; LDB = leaf explant derived from primary bulb regenerants; GA = gelling agent; PGRs = plant growth regulators. The PGR concentrations tested were (I) 4 µM BA (II) 4 µM *m*T (III) 4 µM BA + 5 µM NAA (IV) 4 µM *m*T + 5 µM NAA and (V) PGR-free (control). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; NAA = Naphthalene acetic acid.

Decontaminated plant materials (leaves and bulbs) were inoculated in culture tubes (100 x 25 mm, 40 ml) containing 10 ml Murashige and Skoog (MS) medium **(MURASHIGE and SKOOG 1962)**. **Appendix 1** shows the chemical composition of the MS medium used for the current study. The PGR-medium was supplemented with 30 g/l of sucrose, 0.1 g/l myo-inositol and the pH adjusted to 5.8 with 1M KOH or HCl as required. The medium was solidified with 3 g/l gelrite (Labretoria, Pretoria, South Africa), then autoclaved at 121 °C and 103 kPa for 20 min. The cultures were incubated in 16/8 h light/dark conditions with a photosynthetic photon flux (PPF) of 45 µmol m^{-2} s^{-1} at 25 ± 2 °C. After shoot regeneration (from primary bulb and leaf explants), the resultant leaf materials were used as explants for subsequent experiments. Leaves derived from primary bulb regenerants were denoted as LDB while those from the primary leaf regenerants were coded as LDL **(Fig. 3.1)**.

3.2.3. *In vitro* shoot proliferation using different gelling agents, explant source and plant growth regulators

A 2×2×5 factorial experiment involving gelling agents (agar versus gelrite), explant source (LDB = from primary bulb versus LDL = primary leaf regenerants) and five PGR combinations (including the control) were designed in a randomized manner **(Fig. 3.1)**. Based on trials and previous studies **(BERRIOS et al. 1999; TAYLOR and VAN STADEN 2001b)**, gelrite and agar (Bacteriological agar–Oxoid Ltd., Basingstoke, Hampshire, England) were tested at 3 and 8 g/l, respectively. Both LDB and LDL explants measuring approximately 1 × 1 cm were used for the experiments. The

explants were placed abaxial side down on the medium **(TAYLOR and VAN STADEN 2001b)**.

Media were supplemented with different combinations of cytokinins (CKs) and NAA as follows (I) 4 µM BA (II) 4 µM *m*T (III) 4 µM BA + 5 µM NAA and (IV) 4 µM *m*T + 5 µM NAA. Media solidified with agar or gelrite without any PGR served as a control. One leaf explant was inoculated per culture tube. There were 25 replicates per treatment and the experiment was done twice. The same pH range and growth conditions as described in the preceding section were also applicable during the shoot proliferation experiment. After 10 weeks, growth parameters such as shoot number, shoot length, root number, root length, shoots longer than 5 mm and fresh weight were measured and recorded.

3.2.4. Preparation of extracts for phytochemical quantification

Regenerants from the different treatments described above were oven-dried at 50 °C for five days and ground into fine powders. Ground plant materials were extracted using 50% methanol (MeOH) at 0.1 g per 10 ml in a sonication bath (Julabo GmbH, West Germany) containing ice-cold water for 20 min. The extracts were centrifuged using a Benchtop centrifuge (Hettich Universal, Germany) at 5000 rpm to obtain the supernatant required for the phytochemical content quantification.

3.2.4.1. Determination of iridoid content

Iridoid content was determined using the colourimetric method described by **LEVIEILLE and WILSON (2002)**. In triplicate test tubes, 150 µl of 50% MeOH plant extract were added to 1.35 ml of reagent 1 (82 ml methanol, 8 ml concentrated sulphuric acid and 100 mg vanillin). For the blank, 150 µl of 50% MeOH plant extract was added to 1.35 ml reagent 2 (82 ml methanol and 8 ml concentrated sulphuric acid). The reaction occurred at room temperature and absorbance was read at 538 nm using a UV-visible spectrophotometer. Harpagoside (Extrasynthèse, France) was used as a standard for the calibration curve. Iridoid content in the plant extracts was expressed as mg harpagoside equivalents (HE) per gram DW. Extracts were tested in triplicate and experiment was repeated twice.

3.2.4.2. Determination of condensed tannin content

Condensed tannins were determined using the butanol-HCl assay as described by **MAKKAR et al. (2007)**. Five hundred microlitres of 50% MeOH plant extract were added to 3 ml of the butanol-HCl reagent (95:5, v/v) followed by 100 µl of the ferric reagent (2% ferric ammonium sulphate in 2N HCl). The blank contained 500 µl of 50% MeOH in place of the extract. The reaction solution was mixed using a vortex and left in a water bath at 100 °C for 1 h. The absorbance was measured at 550 nm using a UV-visible spectrophotometer. Cyanidin chloride (Carl Roth GmbH, Germany) was used as a standard for the calibration curve. Condensed tannins were expressed as mg cyanidin

chloride equivalents (CCE) per gram DW. Extracts were tested in triplicate and experiment was repeated twice.

3.2.4.3. Determination of flavonoid content

Flavonoid content was evaluated using the aluminium chloride ($AlCl_3$) colourimetric assay as described by **ZHISHEN et al. (1999)** with modifications **(MARINOVA et al. 2005)**. In triplicate test tubes, 250 µl of 50% MeOH extract were added to 1 ml of distilled water followed by 75 µl of 5% sodium nitrite ($NaNO_2$). After 5 min, 75 µl of 10% $AlCl_3$ and 500 µl of 1 M sodium hydroxide (NaOH) were added sequentially. The reaction mixture was adjusted to 2.5 ml with 600 µl of distilled water. The reaction solution was thoroughly mixed and absorbance measured at 510 nm using a UV-visible spectrophotometer. Catechin (Sigma-Aldrich, USA) was used for calibration and a mixture containing 50% MeOH instead of plant extract was included as a blank. Flavonoid content was presented as mg catechin equivalents (CE) per gram DW. Extracts were tested in triplicate and experiment was repeated twice.

3.2.4.4. Determination of total phenolic content

Total phenolics in the plant extracts were determined using the Folin-Ciocalteu (Folin-C) assay **(MAKKAR et al. 2007)**. In triplicate test tubes, 50 µl of 50% MeOH extracts were added to 950 µl distilled water, followed by the addition of 500 µl Folin-C reagent (1 N) and 2.5 ml of 2% sodium carbonate (Na_2CO_3). A blank consisting of 50% MeOH in place of plant extract was included. The mixture was incubated at room temperature for

40 min and absorbance was recorded at 725 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Gallic acid (Sigma-Aldrich, USA) was used for standard curve calibration. Total phenolic levels were presented as mg gallic acid equivalents (GAE) per gram dry weight (DW). Extracts were tested in triplicate and experiment was repeated twice.

3.2.5. Data analysis

Experiments were conducted in completely randomized designs. The statistical differences between the mean values of agar and gelrite-solidified treatments were determined by subjecting the data to the Student's *t*-test. The analysis was done using SigmaPlot software (version 8.0). Using SPSS (version 16.0) software, mean values of the various treatments were further subjected to analysis of variance (ANOVA). The significance level was determined at $P \leq 0.05$ (*), $P \leq 0.01$(**) and $P \leq 0.001$ (***).

3.3. Results and discussion

3.3.1. Explant decontamination and regeneration frequency

For decontamination frequency from the stock plant, the use of leaves as initial explant source was slightly higher (70 - 75%) than that obtained from primary bulb explants (40 - 50%). Similar lower decontamination frequencies from bulb explants have been documented among members of the Hyacinthaceae **(McCARTAN and VAN STADEN 1998; TAYLOR and VAN STADEN 2001b)**. High frequencies of contamination arising

from using bulbs are often associated with their high load of soil-borne microbes. Following the use of sterile leaf material as the (secondary) explant type, a regeneration frequency ≥ 80% was observed in both LDB and LDL (data not shown). The regenerants from gelrite-solidified media were bigger, more vigorous and healthier than when using agar treatment **(Fig. 3.2)**.

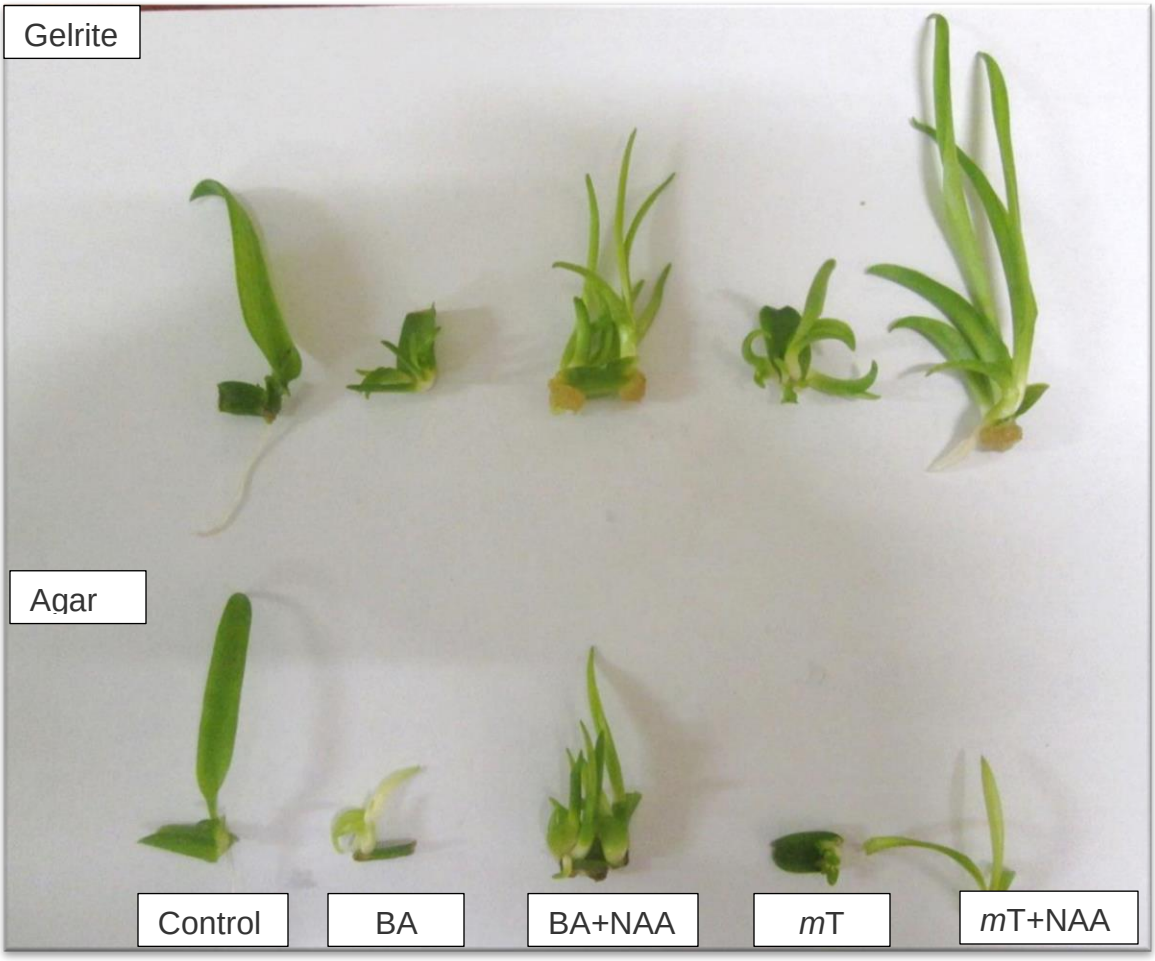


Fig. 3.2: *Eucomis autumnalis* subspecies *autumnalis* regenerants supplemented with diffferent plant growth regulators on either gelrite or agar solidified media after 10 weeks. BA = 6-Benzyladenine; *m*T = *meta*-Topolin; NAA = Naphthalene acetic acid.

3.3.2. Effect of gelling agents on shoot proliferation

Gelrite solidified media generally (apart from BA with NAA treatment for LDB) had a significantly higher number of total and bigger shoots when compared to agar treatment **(Fig. 3.3A and C)**. The highest mean shoot number (c.a 8.5) was observed in gelrite (BA with NAA treatment) while the lowest number (c.a 1.8) of shoots were recorded in agar containing media (LDL in PGR-free and BA as well as LDB in PGR-free treatments). In 7 out of the 10 comparisons, shoot length was consistently higher in gelrite than with agar treatment **(Fig. 3.3B)**. Similar positive effects of gelrite were observed in root (number and length) and fresh weight of *E*. *autumnalis* subspecies *autumnalis* **(Fig. 3.4)**. The current findings reveal the role of applied gelling agents on shoot proliferation in *E*. *autumnalis* subspecies *autumnalis*. Both gelrite and agar have been reported to produce different responses in terms of number of regenerated shoots in micropropagated species. While gelrite was better in some studies **(BARBAS et al. 1993; VERAMENDI et al. 1997; EBRAHIM and IBRAHIM 2000; TSAY et al. 2006; AASIM et al. 2009)**, agar was the preferred choice in other situations **(CORCHETE et al. 1993; BERGER and SCHAFFNER 1995; FATIMA and KHAN 2010; IVANOVA and VAN STADEN 2011)**. In the current study, more shoots were obtained from gelrite compared to agar-containing media. Often, the variation in shoot production between the gelling agents has been partly attributed to the differences in their physicochemical properties. **ARTHUR et al. (2004)** established that numerous gelling agents contain water-soluble root-stimulating (auxin-like) substances which potentially affect growth and development *in vitro*. Agar and gelrite solidified media are known to exhibit different water availability which is primarily due to variation in gel matric potential **(OWNES and**

WOZNIAK 1991). However, it does not necessarily account and fully explain the observed differences in shoot proliferation **(VERAMENDI et al. 1997)**. Perhaps the discrepancies between agar and gelrite media in plant development might result from growth inhibitor effects of agar-inherent impurities **(SCHOLTEN and PIERIK 1998)**. These properties are known to directly affect the availability of water and nutrients that stimulate regeneration of new shoots during micropropagation. Individually, gelling agents and PGRs exhibited the highest levels of significance ($P \leq 0.001$) for all the evaluated growth parameters **(Table 3.1)**.

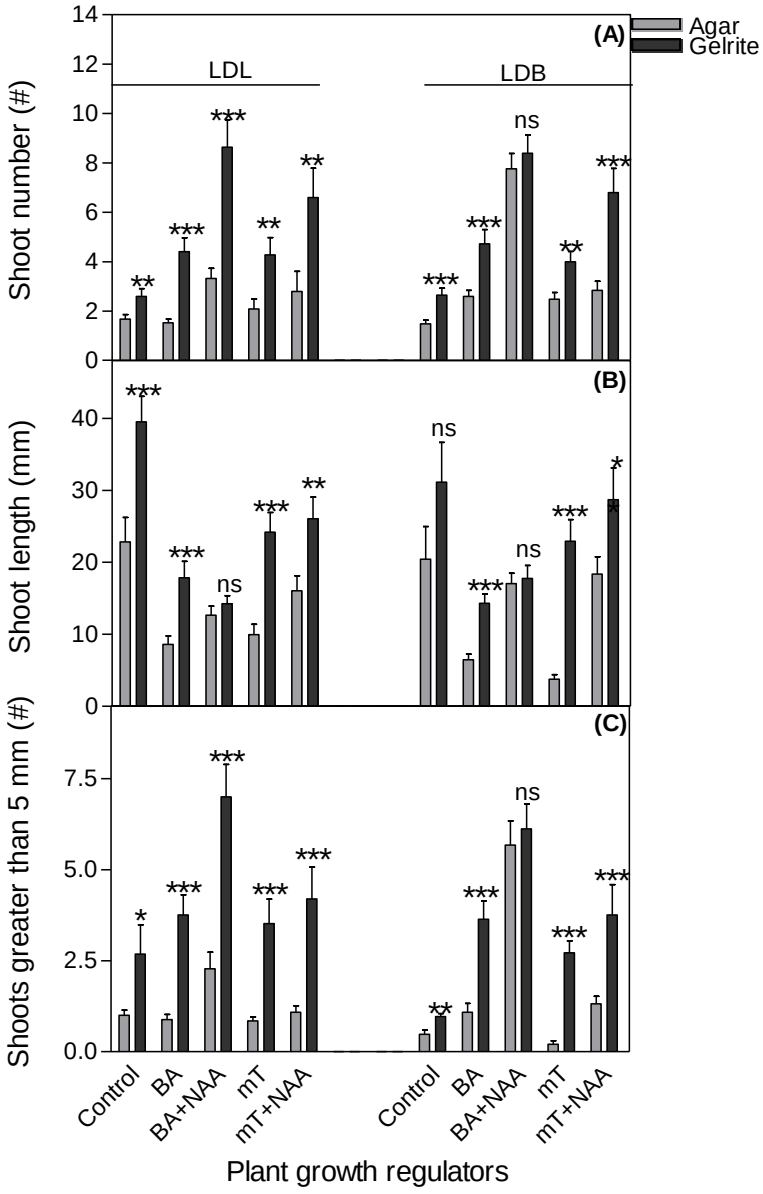


Fig. 3.3: Effect of gelling agents, explant source (LDL = leaf explant derived from primary leaf regenerants; LDB = leaf explant derived from primary bulb regenerants) and plant growth regulators on (A) shoot number, (B) shoot length and (C) shoot greater than 5 mm in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean values ± standard error and n = 50. The levels of significant difference between the mean values of gelling agents were determined using the student's *t*-test. Ns = not significant, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; NAA = Naphthalene acetic acid. Concentrations of BA and *m*T = 4 µM while NAA = 5 µM.

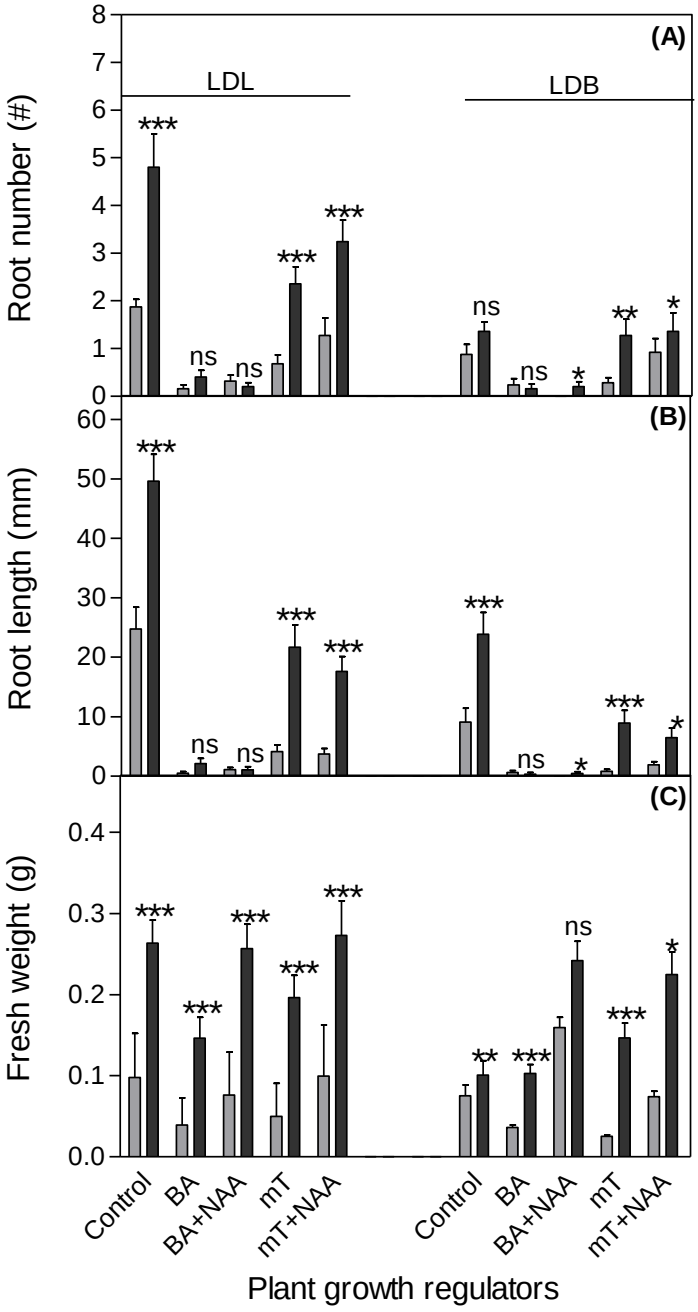


Fig. 3.4: Effect of gelling agents, explant source (LDL = leaf explant derived from primary leaf regenerants; LDB = leaf explant derived from primary bulb regenerants) and plant growth regulators on (A) root number, (B) root length and (C) fresh weight in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean values (± standard error) and n = 50. The levels of significant difference between the mean values of gelling agents were determined using the student's *t*-test. Ns = not significant, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; NAA = Naphthalene acetic acid. Concentrations of BA and *m*T = 4 µM while NAA = 5 µM.

Table 3.1: Analysis of variance (ANOVA) on the effect of gelling agents (GA), explant source (ES) and plant growth regulators (PGRs) as well as their interactions on growth parameters of micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Source of variation	Shoot number			Shoot length			Root number			Root length			Shoot > 5 mm			Fresh weight		
	F-value	*p*-value		F-value	*p*-value		F-value	*p*-value		F-value	*p*-value		F-value	*p*-value		F-value	*p*-value	
GA	83.36	0.000	***	69.77	0.000	***	50.00	0.000	***	87.88	0.000	***	104.32	0.000	***	179.79	0.000	***
ES	4.26	0.040	*	1.19	0.277	ns	50.00	0.000	***	70.16	0.000	***	0.35	0.556	ns	11.92	0.001	***
PGR	40.14	0.000	***	24.26	0.000	***	42.06	0.000	***	104.29	0.000	***	35.63	0.000	***	17.55	0.000	***
GA × ES	4.89	0.027	*	0.19	0.665	ns	14.76	0.000	***	13.40	0.000	***	8.46	0.004	**	13.01	0.000	***
GA × PGR	3.45	0.009	**	4.61	0.001	***	7.58	0.000	***	15.65	0.000	***	1.95	0.102	ns	2.37	0.052	ns
ES × PGR	2.32	0.056	ns	2.09	0.081	ns	10.12	0.000	***	16.65	0.000	***	3.10	0.015	*	4.87	0.001	***
GA × ES × PGR	2.73	0.029	*	0.54	0.708	ns	3.95	0.004	**	1.89	0.111	ns	2.67	0.032	*	1.52	0.196	ns

Ns = Not significant, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

3.3.3. Effect of explant source on shoot proliferation

The importance of the right choice of explant type/source for shoot proliferation in micropropagated plants is well recognized **(IBRAHIM 1994; ISLAM et al. 2005; FIUK and RYBCZYŃSKI 2008)**. In *Eucomis* species, **TAYLOR and VAN STADEN (2001b)** have clearly established that leaf material was most suitable for multiple shoot production compared to bulb material. However, the consequences of using leaf material from different initial explant sources have not been studied until now. With the use of leaf material as a secondary explant type in the current study, the (initial/primary) explant source (LDL and LDB) significantly influenced all the parameters with the exception of shoot length and number of larger shoots **(Table 3.1)**. **McCARTAN and VAN STADEN (1998)** have highlighted the vital role of explant choice as demonstrated in *Merwilla plumbea* (formerly *Scilla natalensis*), another member of the Hyacinthaceae. When comparing agar and gelrite solidified media without PGRs, there was no significant difference in number of shoots from either LDL or LDB explant. In the presence of BA with NAA (agar treatment), there was approximately 2-fold more shoots in LDB compared to LDL regenerants **(Fig. 3.3A)**. The use of leaves as explant source was more effective than the root explant in four *Dieffenbachia* cultivars **(SHEN et al. 2008)**. As postulated by the authors, the differences in responses between the explants may be related to their totipotency. In the absence of PGRs (both agar and gelrite treatments), LDL-derived plantlets had higher number of roots which were significantly longer than LDB regenerants **(Table 3.1; Fig. 3.4A and B).** Although LDL-derived plantlets were significantly bigger than LDB from PGR-free media (gelrite treatment), the fresh weights were generally similar between LDL and LDB in all the remaining treatments **(Fig. 3.4C)**.

3.3.4. Effect of plant growth regulators on shoot proliferation

As an important component of micropropagation, the use of PGRs is often directly associated with an increase in the number of regenerated shoots **(GEORGE 1993)**. Regardless of the gelling agent and explant source, the application of PGRs significantly affected the growth of micropropagated *E*. *autumnalis* subspecies *autumnalis* **(Table 3.1)**. As depicted in **Fig. 3.3A and C**, BA with NAA treatment had the highest shoot proliferation and number of larger (> 5 mm) shoots in LDL (gelrite) and LDB (agar and gelrite) regenerants. In these cases, the mean number of shoots was approximately 4-fold higher in BA with NAA treatment than PGR-free media. Generally, plantlets from PGR-free treatment were either similar or significantly longer (LDL and LDB) than PGR-treated regenerants in both agar and gelrite solidified media **(Fig. 3.3B)**. As evident in both LDL and LDB plantlets **(Fig. 3.4A and B)**, rooting parameters were higher in *m*T and *m*T with NAA treatments than in BA and BA with NAA agar and gelrite solidified media. The better root-stimulating ability of topolins (*m*T in this case) over BA has been observed in several species and partly associated with structural advantages of topolins over BA **(AREMU et al. 2012b)**. Fresh weight was higher when auxins were combined with BA or *m*T compared to the CKs alone for both agar and gelrite solidified media in LDB regenerants **(Fig. 3.4C)**. Although CKs are primarily responsible for shoot production, their (synergistic or antagonistic) interactions with auxins may influence the outcome **(COENEN and LOMAX 1997)**. In the current study, shoot production was enhanced when NAA was combined with CKs (BA or *m*T) compared to the use of CKs alone. In both LDL and LDB regenerants, the number of shoots produced in BA with NAA treatment was approximately 2-fold more than with BA treatment (agar and gelrite) while it was about 1.5-fold more with *m*T with NAA compared to *m*T

treatment from gelrite solidified media **(Fig. 3.3A)**. Similar additive/synergistic effects of auxins have been documented by other researchers **(McCARTAN and VAN STADEN 1998; KOETLE et al. 2010; AMOO and VAN STADEN 2013a)**. As postulated by **NORDSTRÖM et al. (2004)**, the level of active CKs in plants can be regulated by auxins (a more rapid effect) and vice versa, thereby resulting in diverse physiological responses. In addition, post-translational modifications and hormone transport may play important roles in the interactions between auxin and CK **(COENEN and LOMAX 1997)**. However, the underlying mechanism of the interaction remains to be fully elucidated.

3.3.5. Effect of gelling agents on secondary metabolite content

Different *in vitro* factors such as media type and PGRs influence the phytochemicals in regenerated plants **(BAQUE et al. 2010; QUIALA et al. 2012; AMOO and VAN STADEN 2013a)**. The iridoids, condensed tannins, flavonoids and phenolics of *E*. *autumnalis* subspecies *autumnalis* from agar and gelrite solidified media are presented in **Fig. 3.5**. The comparison of agar and gelrite treatments indicates that the iridoid content was generally higher in agar compared to gelrite **(Fig. 3.5A)**. Similarly, the flavonoids were generally higher in *E*. *autumnalis* subspecies *autumnalis* cultured on agar **(Fig. 3.5C)**. There was approximately 3-fold more flavonoids with agar (*m*T treatment derived from LDB) than the gelrite (*m*T treatment derived from LDB) regenerants. In most cases, the levels of condensed tannins and phenolics were generally non-significant between the agar and gelrite treatments. Although studies focusing on the role of gelling agent on phytochemicals are not common, **HENDERSON and KINNERSLEY (1988)** observed lower quantities of anthocyanin in *Daucus carota* grown on media gelled with agar when compared to

corn starch. The varying responses are partly due to the physicochemical properties of the gelling agent used **(DEBERGH 1983)**, which may in turn directly or indirectly affect metabolic pathways of the phytochemicals. During stress, the phenylpropanoid pathway is of critical importance as its products (phenolic compounds) protect the plant against abiotic and biotic factors **(DIXON and PAIVA 1995)**. Thus, the likelihood that gelling agents exerted certain levels of stress in the regenerated plantlets could account for the levels of phytochemicals. Overall, the interaction among gelling agents, explant source and PGRs significantly (in most cases) affected the concentration of the quantified secondary metabolites, with the exception of total phenolics whereby it had relatively lower influences **(Table 3.2)**.

Table 3.2: Analysis of variance (ANOVA) on the effect of gelling agents (GA), explant source (ES) and plant growth regulators (PGR) as well as their interactions on secondary metabolite content in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Source of variation	Condensed tannins			Flavonoids			Iridoids			Phenolics		
	F-value	*p*-value		F-value	*p*-value		F-value	*p*-value		F-value	*p*-value	
GA	16.54	0.000	***	25.35	0.000	***	13.74	0.001	***	2.02	0.163	ns
ES	22.48	0.000	***	7.15	0.000	***	2.97	0.031	*	1.36	0.265	ns
PGR	3.78	0.059	ns	50.59	0.000	***	0.69	0.412	ns	1.94	0.171	ns
GA × ES	7.20	0.000	***	12.88	0.000	***	2.23	0.083	ns	2.98	0.030	*
GA × PGR	0.74	0.394	ns	0.84	0.364	ns	10.01	0.003	**	0.01	0.908	ns
ES × PGR	2.75	0.041	**	19.70	0.000	***	2.27	0.078	ns	4.33	0.005	**
GA × ES × PGR	7.56	0.000	***	6.71	0.000	***	6.04	0.001	***	4.75	0.003	**

Ns = Not significant, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

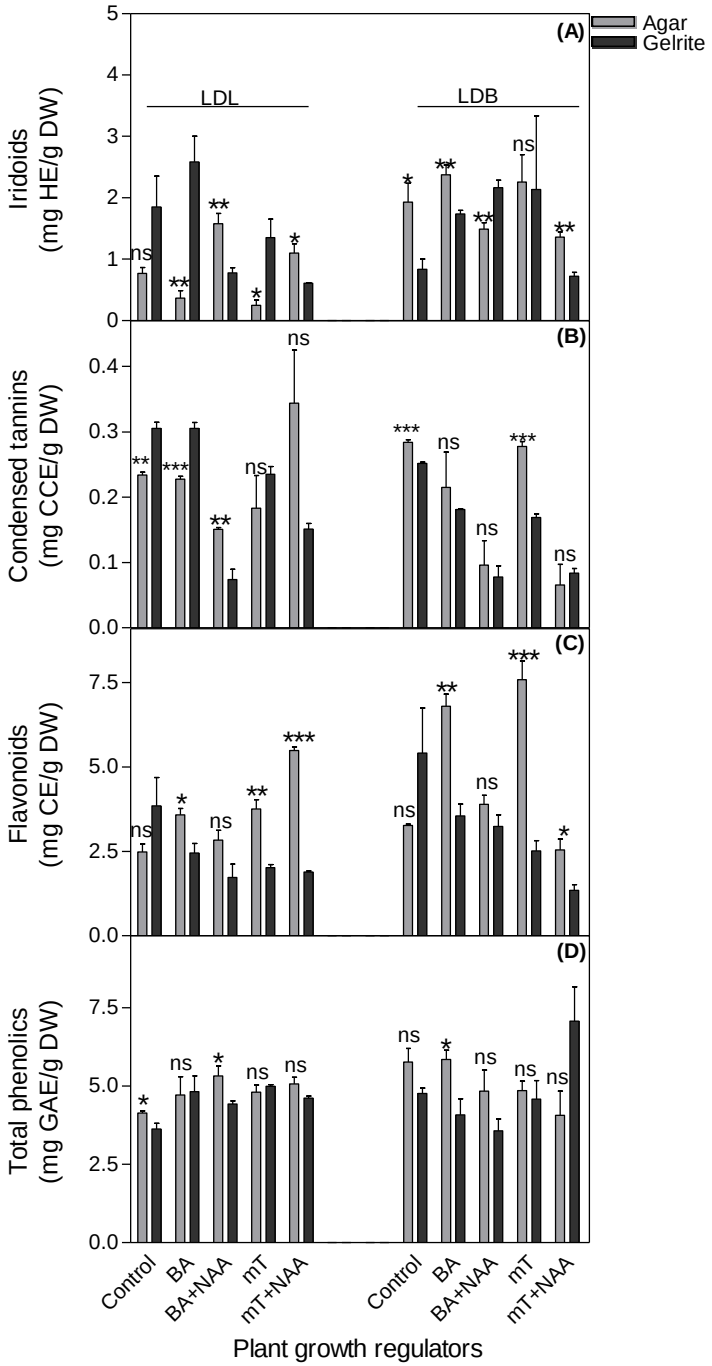


Fig. 3.5: Effect of gelling agents, explant source (LDL = leaf explant derived from primary leaf regenerants; LDB = leaf explant derived from primary bulb regenerants) and plant growth regulators on (A) iridoids, (B) condensed tannins, (C) total flavonoids and (D) total phenolics in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean values ± standard error and n = 6. HE = Harpagoside equivalents; CCE = Cyanide chloride equivalents; CE = Catechin equivalents; GAE = Gallic acid equivalents. The levels of significant difference between the mean values of gelling agents were determined using the student's *t*-test. Ns = not significant, $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; NAA = Naphthalene acetic acid. Concentrations of BA and *m*T = 4 µM while NAA = 5 µM.

3.3.6. Effect of explant source on secondary metabolite content

Globally, there is a steady increase in number of studies evaluating the potential of *in vitro* plant culture systems for the production of desired phytochemicals **(DiCOSMO and MISAWA 1995; RAMACHANDRA RAO and RAVISHANKAR 2002)**. Despite the therapeutic potential of *E*. *autumnalis* subspecies *autumnalis*, there is limited (if any) evidence demonstrating the role of intricate factors such as explant source on levels of accumulated phytochemicals. Based on the current findings, it is logical to expect differences in phytochemical levels due to the observed growth variations from the two explant sources. **Figure 3.5** shows the phytochemical contents in both LDL- and LDB-derived *E*. *autumnalis* subspecies *autumnalis*. Apart from phenolic content (non-significant), the concentration of the quantified secondary metabolites in micropropagated plantlets were significantly affected by the explant source **(Table 3.2)**. From agar solidified media, plantlets derived from LDB (control, BA and *m*T treatments) had remarkably higher iridoids than identical treatments from LDL **(Fig. 3.5A)**. In fact, there was an approximately 6.4-fold (BA) and 9-fold (*m*T) higher iridoid content in plantlets from LDB than from LDL. On the contrary, higher quantity of condensed tannins (3-fold) and flavonoids (2-fold) were quantified in *m*T with NAA regenerants from LDL when compared to LDB in agar solidified media **(Fig. 3.5B and C)**.

3.3.7. Effect of plant growth regulators on secondary metabolite content

While the flavonoid content in plantlets was significantly enhanced by the type of applied PGRs, the phenolic, condensed tannin and iridoid contents were unaffected **(Table 3.2)**. When compared to the control (PGR-free), BA and *m*T (LDB-derived)

treatments significantly (approximately 2-fold) enhanced the level of flavonoids in micropropagated *E*. *autumnalis* subspecies *autumnalis* cultured on agar **(Fig. 3.5C)**. Among the tested PGRs, plantlets (LDB) cultured on agar and supplemented with *m*T had the highest flavonoid content. In addition to the individual effects of the PGRs, the combination of NAA and CKs (BA and *m*T) had both antagonistic (24 out of 32 comparisons) and synergistic (8 out of 32 comparisons) effects on secondary metabolite content in *E*. *autumnalis* subspecies *autumnalis* **(Fig. 3.5)**. In micropropagation, PGRs especially CKs play a vital role in the production of secondary metabolites **(COSTE et al. 2011; SAW et al. 2012)**. The stimulatory effect of CKs has been ascribed to their direct/indirect role on important secondary metabolite biosynthetic pathways **(SAKAKIBARA et al. 2006)**. As highlighted by **RAMACHANDRA RAO and RAVISHANKAR (2002)**, stimulatory and inhibitory effects of auxin on secondary metabolites have been demonstrated in different plant species. The combination of BA and indole-3-butyric acid (IBA) increased phenolic content in *Thymus vulgaris* **(KARALIJA and PARIĆ 2011)** while lower levels of flavonoid content were reported at low concentration of NAA and CKs (*m*T or BA) in *Huernia hystrix* **(AMOO and VAN STADEN 2013a)**. However, combination of IBA and BA in *Mentha piperita* did not enhance secondary metabolite production **(SANTORO et al. 2013)**.

3.4. Concluding remarks

In an attempt to improve growth and phytochemical production, factors affecting micropropagation of *E*. *autumnalis* subspecies *autumnalis* were examined. In addition to the individual effects of gelling agents, explant source and PGRs, these factors interacted in different ways producing various responses. The two gelling

agents responded differently in trade-offs between shoot and secondary metabolite production. There was generally higher shoot production with gelrite while the quantified phytochemicals such as flavonoids and phenolics were more enhanced in agar-supplemented media. In terms of explant source, shoot proliferation and secondary metabolites in regenerants from LDB were better than those from LDL. The importance of PGRs in shoot production was clearly-demonstrated, especially in the presence of a BA with NAA treatment which had the highest shoot production. The levels of secondary metabolites in regenerants were higher with the use of either BA or *m*T compared to their combination with NAA. However, it will be valuable to establish how these aforementioned factors contribute to the overall quality and quantity of the plant after acclimatization. In addition, experiments focusing on approaches that enhance secondary metabolites in gelrite solidified media without drastic reduction in number of shoots will be vital for conservation of the species. The current study articulated the need to fully examine and better understand how *in vitro* culture conditions affect the outcome of micropropagation endeavours.

Chapter 4: The role of plant growth regulators on growth, phytochemical content and antioxidant activity of *Eucomis autumnalis* subspecies *autumnalis*

4. 1. Introduction

Micropropagation involves mass production of somaclones which are genetically and physiologically similar to the mother plant and are easily acclimatized within a relatively short period **(KOZAI et al. 1997; DOBRÁNSZKI and TEIXEIRA DA SILVA 2010)**. The success of micropropagation endeavours is influenced by several intricate physical (e.g. light and temperature) and chemical factors. As a vital chemical component, plant growth regulators (PGRs) regulate various physiological and developmental processes during micropropagation **(GEORGE 1993; GEORGE et al. 2008)**. In an attempt to stimulate or enhance growth *in vitro*, the media are often supplemented with exogenous PGRs, which in turn interact with the endogenous PGRs to produce diverse responses **(GEORGE et al. 2008)**. The regulatory impact of endogenous PGRs is dependent on several factors including (I) the quantity of the available PGRs which is controlled by biosynthesis, degradation and conjugation processes, (II) the location of PGR as mediated by transportation/movement, and (III) receptivity of the receptors and signal-transduction tissues **(DAVIES 2004; GEORGE et al. 2008)**.

Even though a number of growth stimulating substances are used in micropropagation, cytokinins (CKs) and auxins (acting either individually or in combination) are the most important and popular PGRs **(GASPAR et al. 1996; GEORGE et al. 2008)**. Evidently, many aspects of cell growth and cell differentiation as well as organogenesis in micropropagated plants are regulated by an interaction

between exogenously applied CKs and auxins **(KOETLE et al. 2010; AMOO and VAN STADEN 2013a)**. Furthermore, interaction of exogenously applied CKs and auxins has been implicated in the up-regulation of secondary metabolite content in plants **(MEYER and VAN STADEN 1995; AMIT et al. 2005; KARALIJA and PARIĆ 2011)**.

The occurrence of undesired events such as shoot-tip necrosis, hyperhydricity and somaclonal variation is partly associated to the applied PGRs **(BAIRU et al. 2009; ROJAS-MARTÍNEZ et al. 2010; BAIRU et al. 2011)**. In addition, some of the PGRs especially at high concentrations are known to be toxic to the regenerants. In view of the aforementioned limitations of the existing PGRs, there is a continuous effort aimed at identifying new compounds with the ability to stimulate better growth and alleviate *in vitro*-induced physiological disorders **(TARKOWSKÁ et al. 2003)**. The recent biotechnological advances in the field of phytohormones have significantly facilitated the search for new compounds **(STRNAD et al. 1997; TARKOWSKI et al. 2010)**. Thus, a new group of aromatic CKs commonly referred to as topolins has been identified **(STRNAD et al. 1997)**. Topolins have been demonstrated to enhance shoot proliferation, maintain histogenic stability, improve rooting efficiency and alleviate various physiological disorders in micropropagation **(AREMU et al. 2012b)**. Although the positive role of topolins have been reported in a number of micropropagated species, their influence in micropropagated *E*. *autumnalis* subspecies *autumnalis* remains unknown. Furthermore, the effect of combining topolins with auxins in micropropagation remains poorly documented **(AREMU et al. 2012b)**. It is well-known that the optimal environmental and chemical conditions for plant growth and development often vary among species and even genotypes. The

benefits and need for further research especially to optimize the PGR concentrations for shoot proliferation in *Eucomis* species have been highlighted **(AULT 1995; TAYLOR and VAN STADEN 2001b)**. Therefore, the current **Chapter** evaluated the effect of five CKs individually and in combination with an auxin on growth, phytochemical content and antioxidant potential in micropropagated *E*. *autumnalis* subspecies *autumnalis*. Furthermore, the carry-over effect of the applied PGRs on acclimatization competence in *in vitro*-derived *E*. *autumnalis* subspecies *autumnalis* was evaluated.

4.2. Materials and methods

4.2.1. Plant growth regulators and explant source

Apart from the three PGRs (BA, *m*T = *meta*-topolin and NAA = α-naphthalene acetic acid) listed in **Section 3.2.1**, the current experiment included three additional topolins. These were *m*TTHP [*meta*-topolin tetrahydropyran-2-yl or 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine]; Me*m*T [*meta*-methoxytopolin or 6-(3-methoxybenzylamino)purine] and Me*m*TTHP [*meta*-methoxy 9-tetrahydropyran-2-yl topolin or 2- [6-(3-Methoxybenzylamino)-9-(tetrahydropyran-2-yl)purine] **(see Appendix 2)**. Details of the preparation of the topolins have been described previously **(DOLEŽAL et al. 2006; 2007; SZÜCOVÁ et al. 2009)**. Two of the tested CKs (*m*TTHP and Me*m*TTHP) are recently synthesized topolin derivatives and have been tested in only a few plant species prior to the current study **(AREMU et al. 2012b; PODLEŠÁKOVÁ et al. 2012; AMOO et al. 2014)**. Aseptically-obtained leaves derived from primary bulb regenerants as described in **Section 3.2.2** were

subcultured on PGR-free Murashige and Skoog (MS) medium and used for all the experiments in this **Chapter**.

4.2.2. *In vitro* shoot proliferation using different cytokinins

The effect of five CKs (BA, *m*T, Me*m*T, *m*TTHP and Me*m*TTHP) on *in vitro* shoot proliferation was evaluated. Each CK was tested at three concentrations (2, 4 and 6 µM) while the control was CK-free. All the MS (CK-free and CK-treated) media were supplemented with myo-inositol (0.1 mg/ml). Based on the results from shoot proliferation (agar versus gelrite) experiments in **Chapter 3**, media were solidified with gelrite (3 g/l). Three leaf explants (1 × 1 cm) were inoculated in each culture jar (110 x 60 mm, 300 ml volume) containing 30 ml of CK-free or CK-supplemented MS medium. Each treatment had 24 replicates and the experiment was done twice. The cultures were incubated in 16/8 h light/dark conditions with a photosynthetic photon flux (PPF) of 45 µmol m^{-2} s^{-1} at 25 ± 2 °C. After 10 weeks in culture, growth parameters including shoot number, shoot length, root number and root length were measured.

4.2.3. *In vitro* shoot proliferation using different cytokinins and varying concentrations of α-naphthalene acetic acid

Based on the shoot proliferation results from the preceding **Section**, the effect of interaction of CK and NAA was evaluated. Due to the absence of a significant increase in shoot proliferation with an increase in CK concentration, 2 µM CK was used for the current experiment. Using a completely randomized pattern, the experiment was conducted in a 6 × 5 factorial design involving six PGR treatments

(CK-free, BA, *m*T, Me*m*T, *m*TTHP and Me*m*TTHP) and five concentrations of NAA (0, 2.5, 5, 10 and 15 µM). Each treatment had 24 explants and the experiment was done twice. Cultures were grown under the same conditions as stated in **Section 4.2.2**. Similar growth parameters highlighted in **Section 4.2.2** were measured after 10 weeks.

4.2.4. Acclimatization of *in vitro*-derived *Eucomis autumnalis* subspecies *autumnalis*

For comparison purpose, regenerants (n = 15) from PGR-free, CK as well as the combination of CK with NAA at 2.5 and 15 µM were acclimatized. These regenerants were washed free of gelrite and transferred to 7.5 cm diameter pots containing sand:soil:vermiculite (1:1:1, v/v/v) mixture, treated with 1% Benlate® (Du Pont de Nemour Int., South Africa). The regenerants had 2 weeks transition in the mist-house with a misting duration of 10 s at 15 min (80 - 90% relative humidity), day/night temperature of 30/12 ºC and midday PPF of 30 - 90 µmol m^{-2} s^{-1} under natural photoperiod conditions. For a further 14 weeks, the regenerants were maintained in the greenhouse with a day/night temperature of approximately 30/15 ºC, average PPF of 450 µmol m^{-2} s^{-1} and 30 - 40% relative humidity under natural photoperiod conditions. After 4 months, growth parameters including acclimatization survival (%), leaf number, leaf length, root number, root length, bulb diameter and fresh weight were measured. The leaf area was determined using an L1-3100 area meter (Li-Cor Inc., Lincoln, Nebraska, USA).

4.2.5. Phytochemical evaluation of *in vitro* and greenhouse-acclimatized *Eucomis autumnalis* subspecies *autumnalis*

Plant materials from the 10 week-old-*in vitro* **(Section 4.2.3)** and 4 month-old-acclimatized **(Section 4.2.4)** *E*. *autumnalis* subspecies *autumnalis* were harvested. *In vitro* regenerants were assayed as whole plants while the greenhouse grown *in vitro*-derived plants was separated into aerial (leaves) and underground parts (bulbs and roots). The plant materials were oven-dried at 50 ± 2 ºC for 7 days and milled into powder form. Preparation of the extract for phytochemical quantification was done as outlined in **Section 3.2.4**. Iridoid, condensed tannin, flavonoid and phenolic content were expressed as mg harpagoside equivalents (HE), cyanidin chloride equivalents (CCE), catechin equivalents (CE) and gallic acid equivalents (GAE) per g dry weight (DW), respectively. For each experiment, six replicates were evaluated.

4.2.6. Antioxidant evaluation of *in vitro* and greenhouse-acclimatized *Eucomis autumnalis* subspecies *autumnalis*

In vitro (whole plant) and greenhouse (aerial and underground) plant materials were extracted as described in **Section 3.2.4**. The dried extracts were re-suspended in 50% MeOH at 50 mg/ml for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and 12.5 mg/ml for the *βeta*-carotene/linoleic acid antioxidant model systems.

4.2.6.1. DPPH free radical scavenging activity

The DPPH free radical scavenging activity (RSA) of the extract was evaluated as described by **KARIOTI et al. (2004)** with slight modifications **(SHARMA and BHAT**

2009). In Eppendorf tubes, 15 µl plant extract were added to 735 µl of MeOH and 750 µl of DPPH (100 µM) solution. A background solution containing 15 µl of plant extract and 1485 µl of MeOH was used in order to remove absorbance due to extract colour. Ascorbic acid and MeOH were used as positive and negative controls, respectively. The solution was incubated at room temperature for 30 min in the dark and absorbance read at 517 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). The extracts and ascorbic acid were tested at a final concentration of 0.5 mg/ml. Extracts were tested in triplicate and experiment was repeated twice. The free RSA was calculated using the following equation:

$$\text{RSA (\%)} = \left[1-\left(\frac{A_{extract}-A_{background}}{A_{control}}\right)\right] \times 100$$

where $A_{extract}$, $A_{background}$, and $A_{control}$ are the absorbance values of the extract, background and negative control, respectively.

4.2.6.2. *βeta*-carotene/linoleic acid antioxidant model system

βeta-carotene/linoleic acid oxidation inhibitory activity was evaluated as described by **AMAROWICZ et al. (2004)** with slight modification **(MOYO et al. 2010)**. In a brown Schott bottle, 10 mg of *β*-carotene was dissolved in 10 ml chloroform and excess chloroform was evaporated under vacuum leaving a thin film of *β*-carotene. Linoleic acid (200 µl) and Tween 20 (2 ml) were added to the *β*-carotene solution and made to 500 ml with distilled water. The mixture was shaken to form an orange-coloured emulsion. In test tubes, 2.4 ml of the emulsion was added to 100 µl of 50% MeOH extract. The absorbance of the reaction mixture was read at 470 nm immediately and after 1 h incubation at 50 °C. Butylated hydroxytoluene (BHT) and 50% MeOH were used as positive and negative controls, respectively. The extracts and BHT were

tested at a final concentration of 0.5 mg/ml. Extracts were tested in triplicate and experiment was repeated twice. The rate of *β*-carotene bleaching was calculated as follows:

$$\text{Rate of } \beta\text{-carotene} = \left[\ln\left(\frac{A_{t=0}}{A_{t=t}}\right)\right] \times \frac{1}{t}$$

where $A_{t=0}$ absorbance at 0 h, and $A_{t=t}$ absorbance at 1 h. The calculated average rates are used to evaluate the extract antioxidant activity (ANT) and expressed as *β*-carotene bleaching percentage inhibition using the following formula:

$$\text{ANT (\%)} = \left(\frac{R_{control} - R_{extract}}{R_{control}}\right) \times 100$$

where $R_{control}$ and $R_{extract}$ are the average *β*-carotene bleaching rates for negative control and plant extract, respectively.

4.2.7. Data analysis

Experiments were conducted in completely randomized designs. The growth, phytochemical contents and antioxidant activity data were subjected to analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 16.0 Chicago, USA). Where there was statistical significance ($P \leq 0.05$), the mean values were further separated using Duncan's multiple range test.

4.3. Results and discussion

4.3.1. Effect of plant growth regulators on *in vitro* shoot proliferation and greenhouse growth

As shown in **Table 4.1**, mean shoot proliferation was lowest in CK-free (2 shoots/explant) and highest (5.4 shoots/explant) in 4 µM Me*m*TTHP treatments. The observed shoot proliferation range was approximately 2.5-fold higher than values reported for *E*. *autumnalis* subspecies *autumnalis* by **TAYLOR and VAN STADEN (2001b)**. The slight increase in shoot number in CK treatments when compared to CK-free medium suggest that exogenous application of CK is neither a vital requirement for shoot induction nor for proliferation of *E*. *autumnalis* subspecies *autumnalis*. Apart from 2 µM *m*T treatment which is similar to CK-free, the root number of all CK treatments (at the three tested concentrations) was significantly lower than the CK-free regenerants **(Table 4.1)**. Furthermore, the number of roots produced and root length decreased with an increase in the concentration of applied CKs, with exception for *m*TTHP treatment with an increase at 4 µM. Even though it is mostly severe with BA-treated regenerants, high CK concentrations (regardless of the type) can become inhibitory to root growth in micropropagated plants **(WERBROUCK et al. 1996; BAIRU et al. 2008; VALERO-ARACAMA et al. 2010; AMOO et al. 2011)**. As suggested by these authors, exogenous CKs especially when in high concentrations are converted to the irreversible N^7- and N^9-glucoside conjugates, which are biologically inhibitory and cannot be hydrolysed to the active free base form when required for plant growth. In the current study, to an extent, rooting parameters (number and length) were higher with *m*T and *m*TTHP treatments than in BA, Me*m*T and Me*m*TTHP. Unlike BA, the presence of hydroxyl

(-OH) group in topolins allows for formation of *O*-glucosides which are considered to be CK storage forms and rapidly converted to active cytokinin bases when required **(WERBROUCK et al. 1996)**. The presence of methyl (-CH_3) group in the structure of Me*m*T and Me*m*TTHP differentiate them from the other tested topolins (*m*T and *m*TTHP). These structural differences may partly explain the reduced rooting in Me*m*T and Me*m*TTHP treatments.

Table 4.1: Effect of different cytokinin types and concentrations on growth of *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Cytokinin	Conc (μM)	Shoot no (#)	Shoot length (mm)	Root no (#)	Root length (mm)	Fresh weight (g)
Control	0	2.0 ± 0.18 ^{g}	42.7 ± 4.94 ^{a}	2.7 ± 0.49 ^{a}	31.6 ± 3.83 ^{ab}	0.196 ± 0.0317 ^{b}
BA	2	4.8 ± 0.48 $^{a-e}$	9.5 ± 0.80 ^{ef}	0.2 ± 0.16 ^{g}	0.6 ± 0.46 ^{e}	0.119 ± 0.0293 ^{b}
	4	5.3 ± 0.51 ^{ab}	12.0 ± 1.41 $^{c-f}$	0.1 ± 0.07 ^{g}	1.3 ± 0.78 ^{e}	0.225 ± 0.0523 ^{b}
	6	4.6 ± 0.37 $^{a-f}$	7.1 ± 0.55 ^{f}	0.0 ± 0.00 ^{g}	0.0 ± 0.00 ^{e}	0.089 ± 0.0094 ^{b}
*m*T	2	3.9 ± 0.49 $^{a-g}$	19.7 ± 2.60 ^{b}	2.2 ± 0.33 ^{ab}	34.3 ± 4.87 ^{a}	0.111 ± 0.0136 ^{b}
	4	3.9 ± 0.40 $^{a-g}$	15.3 ± 1.74 $^{b-e}$	1.2 ± 0.28 $^{c-e}$	16.2 ± 3.83 ^{cd}	0.109 ± 0.0122 ^{b}
	6	3.4 ± 0.26 $^{e-g}$	17.4 ± 1.82 ^{bc}	1.5 ± 0.23 $^{b-d}$	17.1 ± 3.17 ^{cd}	0.121 ± 0.0325 ^{b}
*m*TTHP	2	3.7 ± 0.53 $^{d-g}$	15.7 ± 1.57 $^{b-e}$	1.9 ± 0.24 ^{bc}	21.4 ± 3.77 ^{c}	0.158 ± 0.0541 ^{b}
	4	3.2 ± 0.23 $^{e-g}$	20.9 ± 1.61 ^{b}	2.0 ± 0.26 ^{bc}	24.4 ± 3.74 ^{bc}	0.105 ± 0.0125 ^{b}
	6	3.0 ± 0.47 ^{fg}	14.1 ± 2.44 $^{b-e}$	1.5 ± 0.25 $^{b-d}$	16.2 ± 2.90 ^{cd}	0.081 ± 0.0112 ^{b}
Me*m*T	2	3.8 ± 0.26 $^{b-f}$	17.2 ± 2.22 $^{b-d}$	1.0 ± 0.18 $^{d-f}$	8.6 ± 2.17 ^{de}	0.187 ± 0.0587 ^{b}
	4	4.4 ± 0.38 $^{a-g}$	9.9 ± 0.79 ^{ef}	0.6 ± 0.16 $^{e-g}$	5.2 ± 1.47 ^{e}	0.097 ± 0.0100 ^{b}
	6	4.4 ± 0.37 $^{a-g}$	14.4 ± 2.29 $^{b-e}$	0.2 ± 0.07 ^{g}	1.3 ± 0.71 ^{e}	0.206 ± 0.0761 ^{b}
Me*m*TTHP	2	4.0 ± 0.47 $^{a-g}$	20.0 ± 2.23 ^{b}	1.4 ± 0.24 ^{cd}	16.5 ± 3.35 ^{cd}	0.122 ± 0.0131 ^{b}
	4	5.4 ± 0.70 ^{a}	10.3 ± 1.31 $^{d-f}$	0.5 ± 0.16 ^{fg}	6.4 ± 2.23 ^{e}	0.124 ± 0.0432 ^{b}
	6	5.2 ± 0.87 $^{a-c}$	17.6 ± 2.54 ^{bc}	0.2 ± 0.08 ^{g}	2.8 ± 1.32 ^{e}	0.457 ± 0.1220 ^{a}

In each column, mean values ± standard error (n = 48) with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). Conc = concentration; BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl.

The absence of any significant effect with an increase in CK concentration on shoot proliferation necessitated the need to evaluate the interaction of CKs with auxin. The interaction between auxin and CK influences several aspects of cellular differentiation and organogenesis in tissue and organ cultures **(COENEN and LOMAX 1997; GEORGE et al. 2008)**. Among auxins, NAA is known to easily move across the cell membrane resulting into its rapid accumulation in the plant cells **(NORDSTRÖM et al. 2004)**. **Figure 4.1** depicts the effect of interaction of different CKs with five concentrations of NAA on shoot and root proliferation in *E*. *autumnalis* subspecies *autumnalis*. When compared to the use of Me*m*T, *m*TTHP and Me*m*TTHP alone, their combination with 5 µM NAA stimulated a higher number of shoots. Although **AULT (1995)** reported an increase in shoot production with the interaction of BA and NAA for *E*. *autumnalis* and *E*. *zambesiaca*, similar interaction had no significant effect on the number of shoots produced in *E*. *autumnalis* subspecies *autumnalis*. These contrasting effects of auxin and CK interaction on members of the genus *Eucomis* may be due to the uniqueness of each plant species and differences in the applied PGR concentrations as well as the endogenous hormone levels.

Although auxins are primarily associated with rooting effects **(GASPAR et al. 1996)**, treatments with NAA (5 - 15 µM) alone yielded significantly higher numbers of shoots than PGR-free treatments **(Fig. 4.1A)**. The ability of NAA (alone) to stimulate shoot production in this species indicates the presence of substantial endogenous CK level which ensured an optimum balance between auxin and CK. Similarly, **CHEESMAN et al. (2010)** reported a significant stimulatory effect of indole-3-butyric acid (IBA) and NAA on bulb production in *E*. *zambasica*.

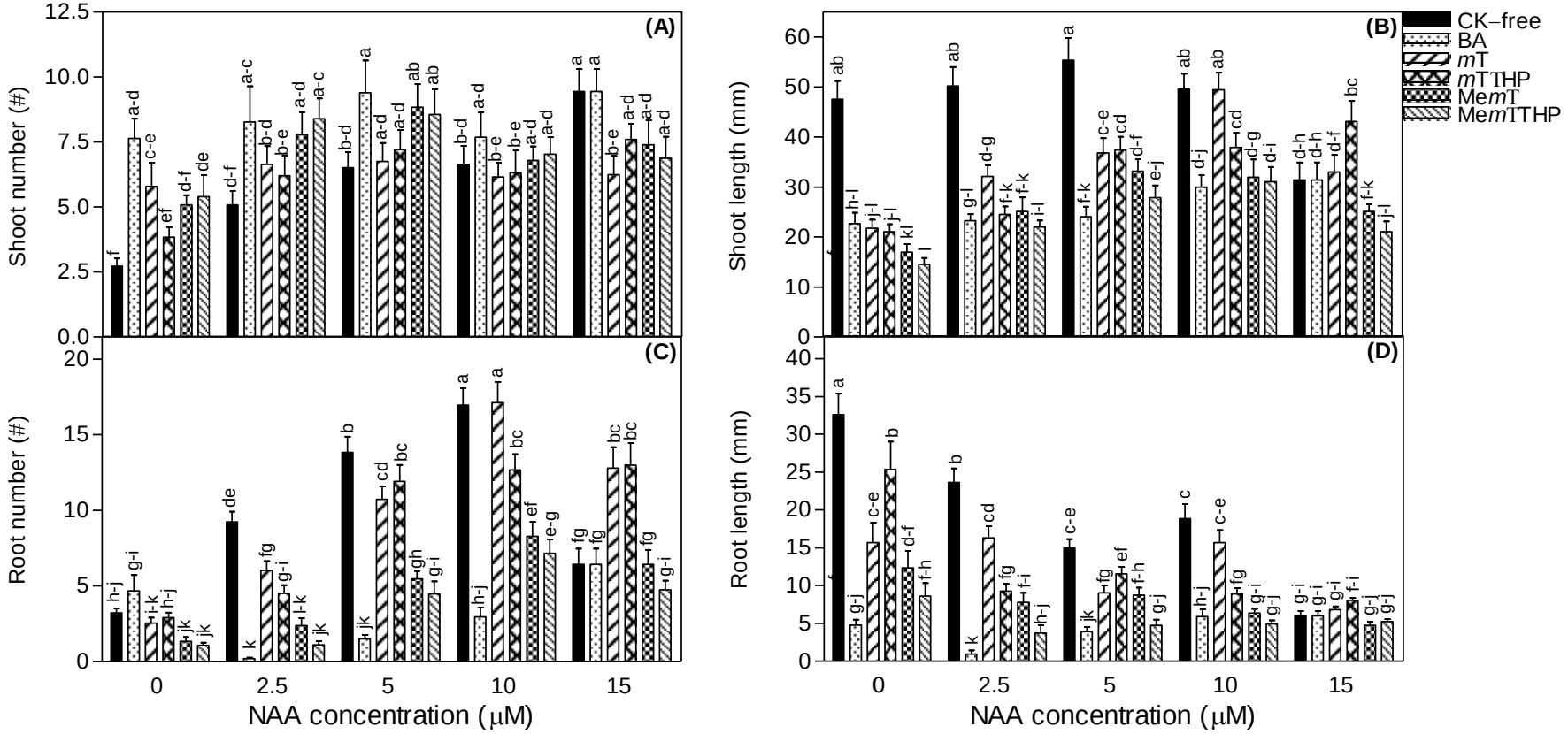


Fig. 4.1: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on (A) shoot number, (B) shoot length, (C) root number and (D) root length in *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. In each graph, bars represent mean values ± standard error (n = 48) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid. The cytokinins were tested at 2 μM.

While the 5 µM NAA treatment produced the longest shoots, 2 µM Me*m*TTHP regenerants were the shortest **(Fig. 4.1B)**. The highest number of roots, approximately 17 roots/explant was obtained in the treatment containing 10 µM NAA alone or in combination with *m*T **(Fig. 4.1C)**. From 2.5 to 10 µM NAA, an increase in root number was observed with CK-free, *m*T and *m*TTHP treatments. Similar enhanced rooting following application of topolins have been reported for several species **(AREMU et al. 2012b)** and ascribed to the increases in acropetal transport of a CK resulting in less accumulation of non-active CK metabolites that could impede rooting **(PODLEŠÁKOVÁ et al. 2012)**. However, increasing concentrations of NAA (particularly at 15 µM) had an inhibitory effect on the root length of the regenerants **(Fig. 4.1D)**. This may be due to an over-production or accumulation of the metabolic products resulting from the high concentration of the exogenously applied auxin **(GEORGE et al. 2008)**.

The overall success of micropropagation lies not only in the production of large numbers of *in vitro* plantlets but also on their survival in field conditions **(HAZARIKA 2006; POSPÍŠILOVÁ et al. 2007)**. Often, tissue culture regenerants may manifest some structural and physiological changes which make them vulnerable to transplantation shock **(KOZAI et al. 1997; AMÂNCIO et al. 1999)**. Even though several intricate factors determine the survival ability of *in vitro* regenerants, the 'carry-over' or 'residual' effect of exogenously applied PGRs has been recognized to be fundamental **(WERBROUCK et al. 1995; VALERO-ARACAMA et al. 2010; AREMU et al. 2012c)**. **Figure 4.2** represents the 4-month-old acclimatized *E. autumnalis* subspecies *autumnalis* derived from cultures containing 15 µM NAA with or without CK. In *m*T and CK-free treatments, there was an estimated 75 - 100%

acclimatization success regardless of the concentration of NAA applied **(Fig. 4.3A)**. Photosynthetic competence which is directly related to the morphology of the leaf is among the crucial factors which affect *ex vitro* survival **(VAN HUYLENBROECK et al. 2000; HAZARIKA 2006; POSPÍŠILOVÁ et al. 2007)**. In this study, the lowest and highest number of leaves was observed in 2.5 µM NAA with *m*TTHP and Me*m*TTHP treatments, respectively **(Fig. 4.3B)**. In terms of the leaf length and area **(Fig. 4.3C and D)**, the most significant effect was obtained with the 15 µM NAA (CK-free) treatment. Addition of NAA especially at 15 µM improved the root growth in most cases **(Fig. 4.4A and B)**.

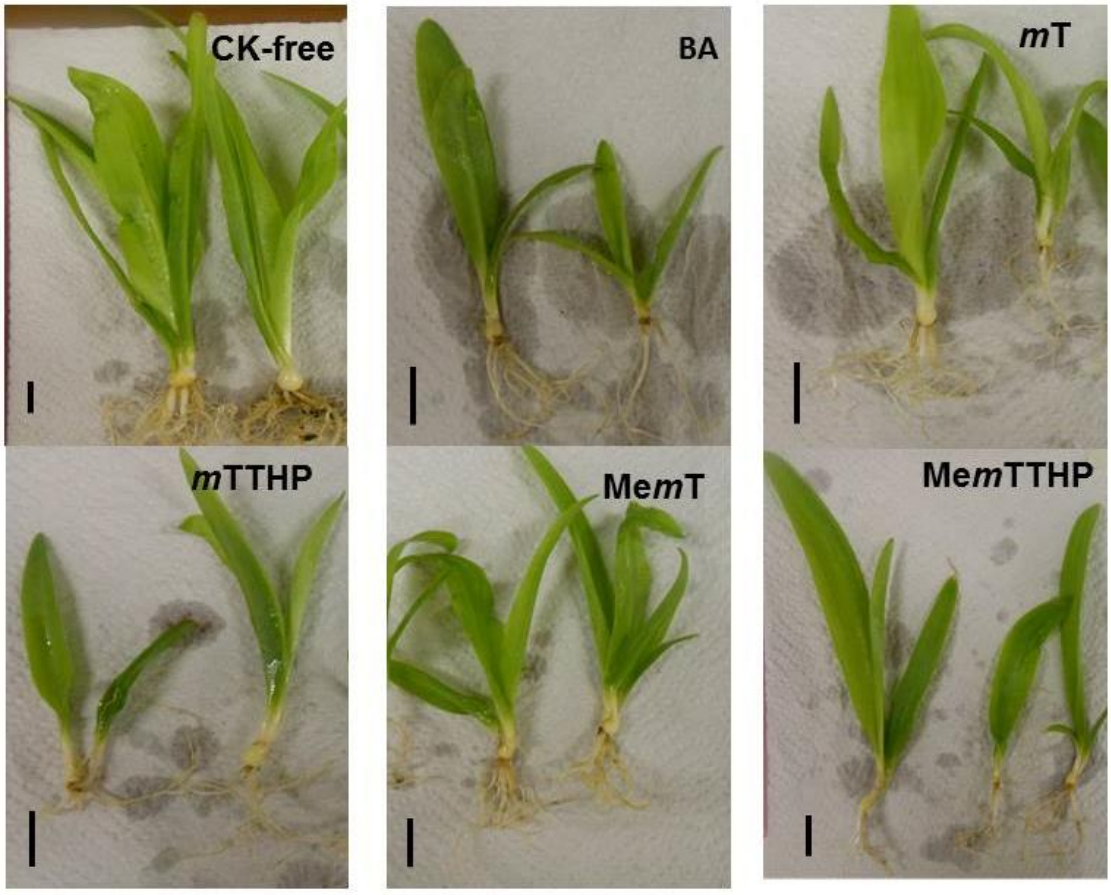


Fig. 4.2: Four-month-old acclimatized *Eucomis autumnalis* subspecies *autumnalis* derived from *in vitro* regenerants supplemented with 15 µM naphthalene acetic acid (NAA) and different cytokinins at 2 µM. BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl. Scale bar = 20 mm.

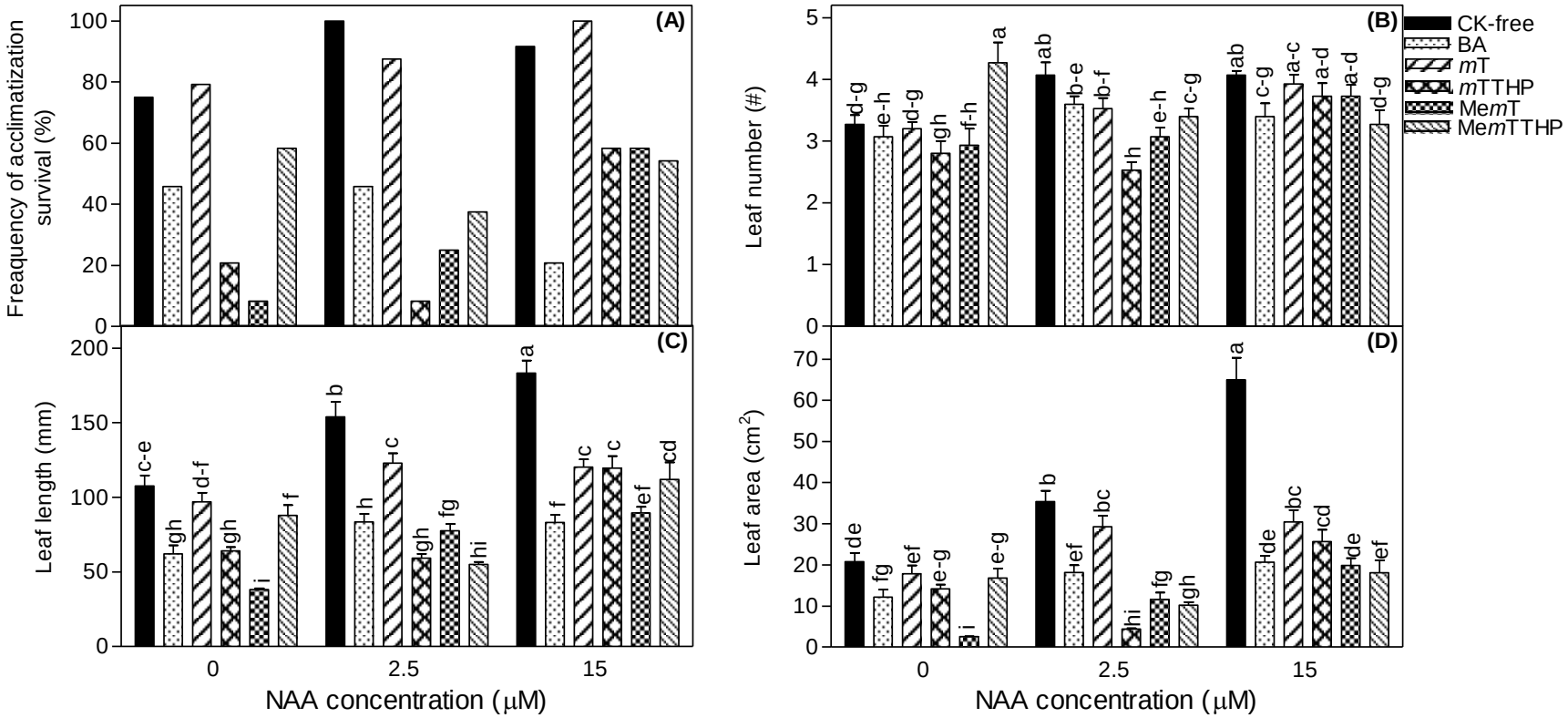


Fig. 4.3: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on (A) frequency of acclimatization survival, (B) leaf number, (C) leaf length and (D) leaf area in 4-month-old acclimatized *Eucomis autumnalis* subspecies *autumnalis*. In each graph, bars represent mean values ± standard error (n = 15) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid. All the cytokinins were tested at 2 µM.

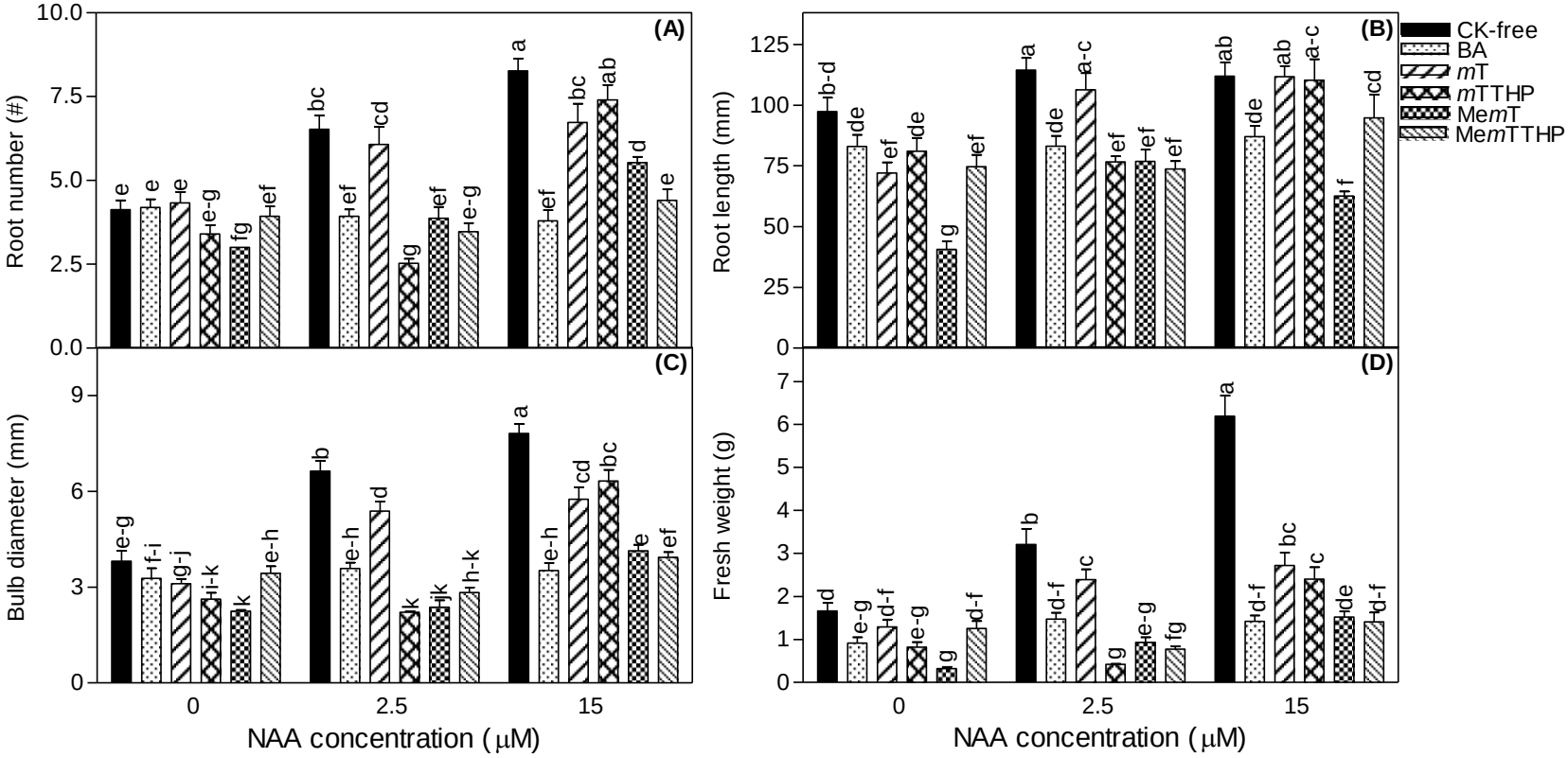


Fig. 4.4: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on (A) root number, (B) root length, (C) bulb diameter and (D) fresh weight of 4-month-old greenhouse acclimatized *Eucomis autumnalis* subspecies *autumnalis*. In each graph, bars represent mean values ± standard error (n = 15) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid. All the cytokinins were tested at 2 μM.

Furthermore, a similar stimulatory 'carry-over' effect of NAA was demonstrated in bulb diameter and fresh weight **(Fig. 4.4C and D)** whereby plants derived from a treatment containing 15 µM NAA alone had the biggest bulb and highest fresh weight. The presence and functionality of roots significantly contribute to survival of micropropagated plants **(HAZARIKA 2006)**. While PGRs such as CK and ethylene are partly associated with rooting, auxins remain the primary signalling PGR **(MALÁ et al. 2009)**. It was evident that NAA enhance rooting *in vitro* **(Fig. 4.1)**, thus allowing for easier establishment and acclimatization upon transferal to the greenhouse, which inevitably explains the enhanced *ex vitro* growth of NAA-derived *E*. *autumnalis* subspecies *autumnalis*.

Although BA is the most commonly used CK for micropropagation of *Eucomis* species **(AULT 1995; McCARTAN and VAN STADEN 1995; TAYLOR and VAN STADEN 2001b)**, there is increasing evidence of its negative (carry-over) effects during acclimatization for several micropropagated species **(AREMU et al. 2012b)**. At equimolar CK concentration without NAA, BA-derived plants were similar to topolins and CK-free treatments in most cases **(Fig. 4.3 and 4.4)**. An exception was the better survival (%) and longer leaf in *m*T, Me*m*TTHP and CK-free plants when compared to BA treatment **(Fig. 4.3A and C)**. Thus, the use of topolins had minimal acclimatization benefits when compared to BA treatment in this species. The observed reduced survival and growth **(Fig.4.3 and 4.4)** in Me*m*T-treated plants when compared to CK-free plants suggests potential inhibitory effects of the applied CK on subsequent *ex vitro* growth and survival.

When compared to *m*T and *m*TTHP, application of BA with NAA was less effective for some of the growth parameters of *E*. *autumnalis* subspecies *autumnalis*. For instance, BA with 15 µM NAA treatment had lower survival, smaller leaves (length), reduced roots number, smaller bulbs and fresh weight **(Fig. 4.3 and 4.4)**. However, not all the topolin interactions with NAA was superior to BA as Me*m*T and Me*m*TTHP with 15 µM NAA treatments were mostly identical to the BA-derived plants. Based on the current findings, it appears as if exogenous application of NAA is more important than CKs (regardless of the types) during micropropagation and subsequent acclimatization of *E*. *autumnalis* subspecies *autumnalis*.

4.3.2. Effect of plant growth regulators on phytochemical contents of *in vitro* regenerants and acclimatized *Eucomis autumnalis* subspecies *autumnalis* plants

The importance of the quality and quantity of phytochemicals in micropropagated medicinal plant species has become well-recognised globally **(DÖRNENBURG and KNORR 1995; SAVIO et al. 2012; SZOPA et al. 2013; SZOPA and EKIERT 2014)**. One of the factors known to influence phytochemical levels in plants is the type and concentration of exogenously supplied PGRs **(RAMACHANDRA RAO and RAVISHANKAR 2002; MATKOWSKI 2008)**. The effect of applied PGRs on the concentrations of secondary metabolites in the micropropagated *E*. *autumnalis* subspecies *autumnalis* is presented in **Fig. 4.5***.* Regenerants derived from 5 µM NAA with *m*T had the highest (1.886 mg HE/g DW) iridoid content while all the other treatments were generally low (≤ 1 mg HE/g DW) **(Fig. 4.5A)**. Although NAA alone had no remarkable influence on iridoid content, its combination (at 2.5 to 10 µM) with

Me*m*T significantly increased the level of iridoids in the regenerants. The highest condensed tannin concentration (0.435 mg CCE/g DW) was elicited with 2.5 µM NAA and *m*TTHP treatment **(Fig. 4.5B)**. Shoots regenerated from CK (*m*T, *m*TTHP) alone or in combination with NAA (2.5 and 5 µM) had a significantly increased condensed tannin content in comparison to PGR-free medium. These findings suggest a possible synergetic interaction of NAA (2.5 to 5 µM) with CKs on accumulated iridoids and condensed tannins in regenerated *E*. *autumnalis* subspecies *autumnalis*. As demonstrated in the current study, the observed variations in phytochemical levels from different CK treatments and interaction with auxins have been reported by other researchers **(LIU et al. 2007; COSTE et al. 2011; BASKARAN et al. 2012; AMOO and VAN STADEN 2013a)**. An explanation for these diverse effects may have resulted from inherent differences in the structure of the PGRs and how they influence the phytochemical biosynthetic pathways.

Addition of NAA had low or no stimulatory effect on the level of flavonoids and total phenolics in the majority of the treatments **(Fig. 4.5C and D)**. In both cases, PGR-free regenerants accumulated the highest level of flavonoids and total phenolics. These reductions in phytochemical (phenolics in this case) in the presence of PGRs have been documented in some micropropagated plants. For example, CK-free *Tectona grandis* and *Aloe arborescens* had a significantly higher concentration of phenolics when compared to BA-treated *T*. *grandis* **(QUIALA et al. 2012)** and *m*TTHP- or benzyladenine riboside-treated *A*. *arborescens* **(AMOO et al. 2014)**. As postulated by the authors, the presence of PGRs (especially at higher concentration) may have exerted some inhibitory effect on the phenolic biosynthetic pathways.

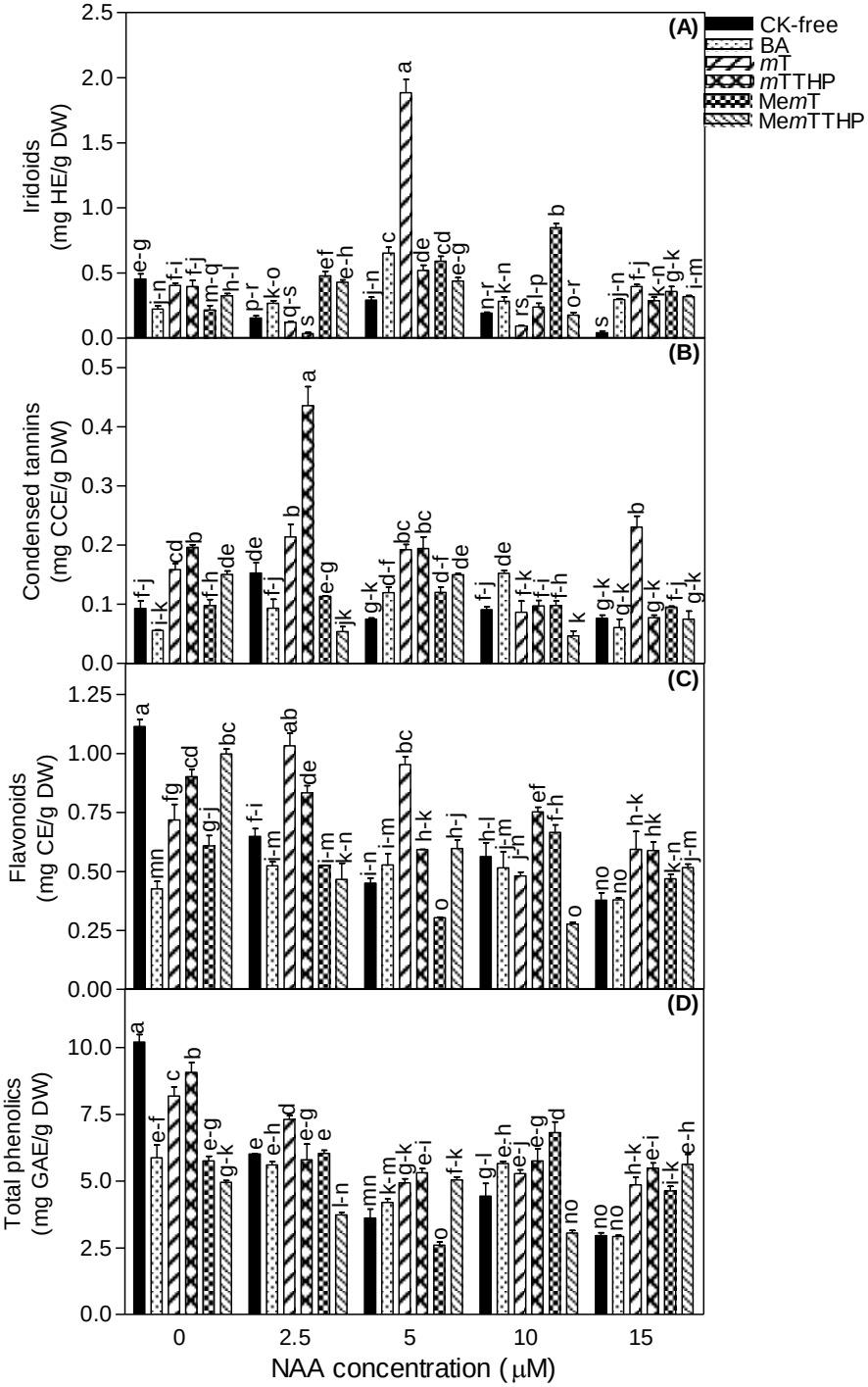


Fig. 4.5: Effect of different cytokinins with naphthalene acetic acid (NAA) concentrations on (A) iridoids, (B) condensed tannins, (C) flavonoids and (D) total phenolics in *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. In each graph, bars represent mean values ± standard error (n = 6) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid. The cytokinins were tested at 2 μM.

Although there is an ever increasing number of studies evaluating the role of *in vitro* culture systems on the production of phytochemicals, information pertaining to the possibility of changes in the chemical content/composition of micropropagated plants after acclimatization are scarce. Nevertheless, such studies allow for elucidation and manipulation of phytochemicals of interest especially at harvest stage **(LIU et al. 2004; NUNES et al. 2009; AREMU et al. 2013)**. Secondary metabolites in the acclimatized *E*. *autumnalis* subspecies *autumnalis* were quantified and compared on the basis of aerial (leaves) and underground (bulbs and roots) parts **(Fig. 4.6)**. It is noteworthy that the acclimatized plants had several fold more secondary metabolites (with exception to the condensed tannins) when compared to similar treatments from the *in vitro* regenerants **(Fig. 4.5 and 4.6)**. In a similar manner, **LIU et al. (2004)** observed a significantly higher flavonoid contents in the tissues of mature greenhouse-grown *Artemisia judaica* than the *in vitro* regenerants. As hypothesized by some researchers **(AHMAD et al. 2013; AREMU et al. 2013)**, age effect may have been the main contributing factor to these observations. Higher levels of iridoids were observed in the aerial parts compared to the underground parts, with the exception of the BA treatment having higher iridoid contents in the underground parts **(Fig. 4.6A and B)**. From a conservation perspective, these findings are valuable as it implies that the aerial parts can serve as alternative sources of (bioactive) phytochemicals mainly sourced from the underground parts **(VAN STADEN et al. 2008)**. Although *m*TTHP treatment had the highest level of condensed tannins, increasing concentrations of NAA significantly reduced the condensed tannins in the aerial parts **(Fig. 4.6C)**. In the underground parts, the highest (0.374 mg CCE/g DW) condensed tannin content was produced in 2.5 µM NAA with Me*m*TTHP treatment **(Fig. 4.6D)**. In both plant parts evaluated, the highest

level (c.a 6 mg CE/g DW) of flavonoids was observed in 2.5 µM NAA with *m*TTHP treatment **(Fig. 4.6E and F)**. Apart from the 2.5 µM NAA with *m*T treatment with higher total phenolics in the underground parts, the aerial parts generally had higher or similar phenolic levels as compared to those quantified in the underground parts **(Fig. 4.6G and H)**. As established in the current study, CK and auxin treatments have been reported to individually and interactively have a significant carry-over effect on phytochemical production in *Aloe arborescens* **(AMOO et al. 2013)** and *Merwilla plumbea* **(AREMU et al. 2013)**.

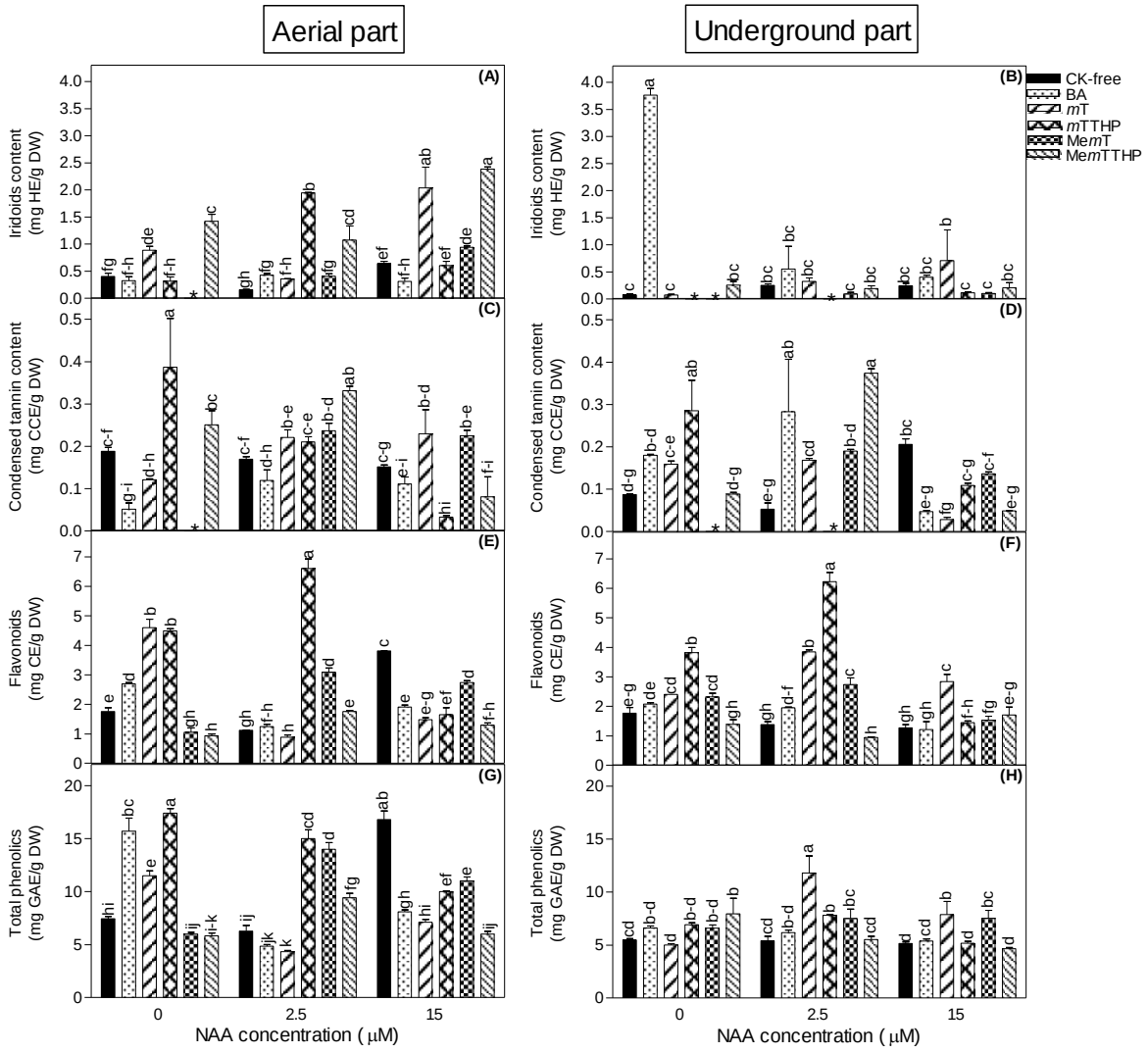


Fig. 4.6: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on (A and B) iridoids, (C and D) condensed tannins, (E and F) flavonoids and (G and H) total phenolics of 4-month-old greenhouse-acclimatized *Eucomis autumnalis* subspecies *autumnalis*. In each graph, bar represents mean values ± standard error (n = 6) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid, * = not tested. All the cytokinins were tested at 2 μM.

4.3.3. Effect of plant growth regulators on antioxidant potential of *in vitro* regenerants and acclimatized *Eucomis autumnalis* subspecies *autumnalis* plants

The potential of *in vitro* plant culture systems for the production of an enormous variety of antioxidant compounds has been recognized **(MATKOWSKI 2008)**. Most *in vitro* antioxidant tests are easy, affordable and allows for high throughput screening, providing a motivation for the evaluation of antioxidant activity in *E*. *autumnalis* subspecies *autumnalis*. Two test systems with different antioxidant mechanisms were used in order to accommodate for complexities involved in antioxidant processes **(HUANG et al. 2005)**. Using extracts from the *in vitro* regenerants, treatment with 5 µM NAA (DPPH assay) and BA (*β*-carotene assays) treatments elicited the highest antioxidant activity **(Table 4.2)**. Generally, the extracts demonstrated better antioxidant activity in the *β*-carotene test system compared to the DPPH free-radical assay. For instance, plant extracts from 15 µM NAA as well as 2.5 or 15 µM NAA with Me*m*TTHP had approximately 4-fold higher antioxidant activity in the *β*-carotene test system compared to the DPPH assay. Conversely, three of the treatments (2.5 µM NAA, 5 µM NAA and 5 µM NAA with BA) had better antioxidant activity in DPPH compared to the *β*-carotene assay. Based on the mechanisms of antioxidant test systems **(AMAROWICZ et al. 2004; HUANG et al. 2005)**, the current findings suggest that the antioxidant principles in *in vitro* regenerated *E*. *autumnalis* subspecies *autumnalis* are more favourable towards hydrogen atom transfer reactions (*β*-carotene assay which involves inhibition of lipid peroxidation) than single electron transfer reactions (DPPH assay).

In the absence of NAA, CK-derived regenerants had a significant higher antioxidant activity (*β*-carotene assay) compared to the PGR-free treatment **(Table 4.2)**. Although NAA treatments (especially 2.5 to 10 µM) had similar antioxidant activity as the PGR-free, combination of NAA (10 and 15 µM) and topolins (*m*TTHP and Me*m*T) significantly improved the antioxidant activity (*β*-carotene assay) when compared to the use of the CK or NAA alone. As demonstrated in other studies **(UCHENDU et al. 2011; HAZARIKA and CHATURVEDI 2013; AMOO et al. 2014)**, the current findings further emphasized the vital role of exogenously applied PGRs (types and concentration) on the resultant antioxidant potential of *in vitro* regenerants.

Table 4.2: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on the antioxidant activity of *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Treatment		Antioxidant activity (%)	
Cytokinin (2 µM)	NAA Conc (µM)	DPPH free radical scavenging	*Beta* -carotene linoleic acid model
Cytokinin-free	0	26.4 ± 2.41 $^{d-h}$	38.6 ± 3.01 ^{mn}
	2.5	37.5 ± 0.20 ^{b}	33.5 ± 2.46 ^{no}
	5	55.2 ± 4.28 ^{a}	45.0 ± 0.73 $^{j-n}$
	10	34.5 ± 1.89 $^{b-d}$	40.4 ± 3.29 $^{l-n}$
	15	15.2 ± 0.86 ^{ij}	52.7 ± 2.19 $^{g-k}$
BA	0	30.2 ± 2.48 $^{b-f}$	87.5 ± 2.96 ^{a}
	2.5	32.3 ± 5.02 $^{b-e}$	79.9 ± 9.46 $^{a-c}$
	5	26.4 ± 3.66 $^{d-h}$	18.6 ± 0.93 ^{p}
	10	27.4 ± 4.17 $^{c-g}$	73.3 ± 6.06 $^{b-d}$
	15	36.8 ± 3.18 ^{b}	61.4 ± 0.04 $^{e-h}$
m T	0	23.2 ± 3.02 $^{e-i}$	57.5 ± 0.91 $^{f-i}$
	2.5	37.4 ± 1.80 ^{b}	54.9 ± 2.34 $^{f-k}$
	5	20.2 ± 0.97 $^{g-j}$	26.0 ± 1.59 ^{op}
	10	23.7 ± 1.88 $^{e-i}$	56.2 ± 4.70 $^{f-j}$
	15	27.8 ± 2.39 $^{c-g}$	57.1 ± 1.53 $^{f-j}$
m TTHP	0	52.1 ± 5.61 ^{a}	66.4 ± 6.59 $^{d-f}$
	2.5	24.9 ± 1.90 $^{e-h}$	43.5 ± 3.81 $^{k-n}$
	5	18.4 ± 1.24 $^{g-j}$	39.9 ± 0.75 ^{mn}
	10	49.2 ± 0.68 ^{a}	80.5 ± 6.07 $^{a-c}$
	15	50.0 ± 2.26 ^{a}	83.3 ± 2.17 ^{ab}
Me*m* T	0	27.0 ± 3.33 $^{d-h}$	47.0 ± 6.32 $^{i-m}$
	2.5	17.6 ± 0.78 $^{h-j}$	55.6 ± 2.59 $^{f-j}$
	5	21.7 ± 1.62 $^{f-j}$	39.6 ± 0.50 ^{mn}
	10	21.5 ± 2.32 $^{f-j}$	59.8 ± 0.92 $^{e-h}$
	15	46.7 ± 4.00 ^{a}	64.2 ± 1.06 $^{d-g}$
Me*m* TTHP	0	31.3 ± 2.81 $^{b-e}$	55.3 ± 2.49 $^{f-k}$
	2.5	12.8 ± 0.63 ^{j}	51.7 ± 4.88 $^{h-l}$
	5	36.3 ± 3.36 ^{bc}	49.8 ± 0.49 $^{h-m}$
	10	25.3 ± 2.68 $^{d-h}$	71.4 ± 2.02 $^{c-e}$
	15	15.2 ± 1.81 $^{i-j}$	56.0 ± 2.44 f-j
Positive controls		Ascorbic acid = 97.6 ± 1.88	Butylated hydroxytoluene = 98.8 ±0.98

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's multiple range test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = Naphthalene acetic acid. All extracts and positive controls were evaluated at final concentration of 0.5 mg/ml.

Despite the increase in number of recent studies **(GARCÍA-PÉREZ et al. 2012; ZAYOVA et al. 2012; AMOO et al. 2013; AREMU et al. 2013)**, the importance of better understanding the general physiology and series of events involved during and after micropropagation of valuable medicinal plants cannot be over-emphasized. Such information especially the pharmacological activity of acclimatized plant is vital from a conservation perspective. **Table 4.3** shows the antioxidant activity of extracts from aerial and underground parts of the acclimatized *E*. *autumnalis* subspecies *autumnalis*. It is noteworthy that the antioxidant activity (mainly DPPH assay) elicited in the 4-month-old acclimatized material were higher (in the aerial part) when compared to similar treatments from *in vitro* regenerants. In contrast, **GARCÍA-PÉREZ et al. (2012)** reported a 28% increase in antioxidant activity of *in vitro Poliomintha glabrescens* when compared to the wild type and acclimatized plants. It was shown that 5 month-old greenhouse-grown *Artemisia judaica* had a significantly higher antioxidant activity when compared to the 3 month-old *in vitro* regenerants **(LIU et al. 2004)**. The type of CK and plant parts investigated significantly influenced the level of antioxidant activity in *in vitro* and acclimatized *Merwilla plumbea* **(AREMU et al. 2013)**. In the current study, extracts from the aerial parts had better DPPH free-radical scavenging activity than the underground parts in all the treatments. Although PGR carry-over effects had no significant influence (when comparing any of the treatments to the control) on DPPH free-radical scavenging activity in aerial parts, 15 µM NAA with Me*m*TTHP treatment had about 2.4-fold higher antioxidant activity than the control (PGR-free) in underground parts. In the *β*-carotene test system, the highest antioxidant activity was observed in 2.5 µM NAA and 15 µM NAA with Me*m*T treatments for the aerial and underground parts, respectively **(Table 4.3)**.

Even though *in vitro* plants possess the possibility of producing standardized phytochemicals (with pharmacological properties), independent from environmental factors, the dynamics and accumulation of these compounds may be tilted under *ex vitro* conditions. According to **AMOO et al. (2013)**, the type of PGR had a significant effect on antioxidant activity in tissue culture-derived *A*. *arborescens* after 2 months *ex vitro* growth. As a quality control measure, when compared to naturally-grown *Pelargonium sidoides*, 1-year-old greenhouse (*in vitro*-derived regenerants) plants exhibited similar antioxidant activity **(MOYO et al. 2013)**. Based on this evidence, it therefore, follows that the acclimatized *E*. *autumnalis* subspecies *autumnalis* have the potential to exhibit similar pharmacological activities as the wild population.

Table 4.3: Effect of combining different cytokinins with naphthalene acetic acid (NAA) concentrations on the antioxidant activity in 4-month-old acclimatized *Eucomis autumnalis* subspecies *autumnalis.*

Treatment		DPPH free-radical scavenging (%)		*Beta* -carotene linoleic acid model (%)	
Cytokinin (2 µM)	NAA Conc (µM)	Aerial	Underground	Aerial	Underground
Cytokinin-free	0	95.8 ± 1.46 ^{a}	23.1 ± 1.59 ^{hi}	83.5 ± 0.49 $^{a-c}$	65.9 ± 3.20 $^{b-e}$
	2.5	90.6 ± 0.54 ^{ab}	22.8 ± 1.64 ^{hi}	87.5 ± 1.56 ^{a}	66.2 ± 1.23 $^{a-e}$
	15	96.1 ± 1.01 ^{a}	21.5 ± 3.20 ^{i}	83.7 ± 6.21 $^{a-c}$	59.8 ± 2.86 $^{d-f}$
BA	0	90.8 ± 1.49 ^{ab}	36.0 ± 2.31 ^{ef}	54.5 ± 2.24 ^{e}	70.8 ± 3.38 $^{a-d}$
	2.5	95.2 ± 1.37 ^{a}	29.4 ± 0.52 $^{f-h}$	59.7 ± 1.37 ^{e}	67.6 ± 2.81 $^{a-e}$
	15	88.8 ± 1.43 ^{ab}	44.1 ± 1.88 ^{cd}	78.3 ± 2.93 $^{b-d}$	64.8 ± 1.38 $^{b-e}$
m T	0	91.5 ± 0.54 ^{ab}	27.1 ± 2.59 $^{g-i}$	86.4 ± 1.17 ^{ab}	60.0 ± 2.55 $^{d-f}$
	2.5	97.1 ± 0.88 ^{a}	20.9 ± 1.77 ^{i}	76.0 ± 3.96 ^{cd}	56.8 ± 5.32 ^{ef}
	15	93.3 ± 0.29 ^{ab}	12.9 ± 2.14 ^{j}	72.4 ± 4.90 ^{d}	66.1 ± 3.19 $^{a-e}$
m TTHP	0	94.4 ± 0.61 ^{a}	41.8 ± 1.95 ^{de}	61.3 ± 3.19 ^{e}	29.0 ± 3.58 ^{g}
	2.5	67.4 ± 0.40 ^{d}	nd	nd	nd
	15	66.9 ± 6.48 ^{d}	50.7 ± 3.95 $^{a-c}$	57.8 ± 3.38 ^{e}	50.8 ± 3.89 ^{f}
Me*m* T	0	nd	nd	nd	nd
	2.5	79.2 ± 4.58 ^{c}	52.3 ± 2.21 ^{ab}	nd	73.3 ± 6.56 $^{a-c}$
	15	91.3 ± 1.02 ^{ab}	30.8 ± 1.55 ^{fg}	54.2 ± 0.57 ^{e}	78.0 ± 2.89 ^{a}
Me*m* TTHP	0	88.4 ± 2.25 ^{ab}	29.3 ± 5.18 $^{f-h}$	57.6 ± 3.95 ^{e}	73.8 ± 5.15 ^{ab}
	2.5	84.9 ± 6.11 ^{bc}	46.8 ± 0.86 $^{b-d}$	57.3 ± 1.07 ^{e}	61.6 ± 6.12 $^{c-f}$
	15	90.1 ± 1.66 ^{ab}	54.7 ± 0.94 ^{a}	54.6 ± 3.25 ^{e}	72.6 ± 2.71 $^{a-c}$
Positive controls		Ascorbic acid = 97.6 ± 1.88		Butylated hydroxytoluene = 98.8 ±0.98	

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's multiple range test (DMRT). BA = 6-Benzyladenine; *m*T = *meta*-Topolin; *m*TTHP = *meta*-Topolin tetrahydropyran-2-yl; Me*m*T = *meta*-Methoxytopolin; Me*m*TTHP = *meta*-Methoxytopolin tetrahydropyran-2-yl; NAA = α-Naphthalene acetic acid; nd = not determined. All extracts and positive controls were evaluated at final concentration of 0.5 mg/ml

4.4. Concluding remarks

The current findings provide an improved micropropagation protocol for *E*. *autumnalis* subspecies *autumnalis* with emphasis on the exogenously applied PGRs. Depending on the overall objectives, topolins can serve as suitable alternatives for conventional/commonly used BA for the species. Even though CKs are required for enhanced shoot proliferation, there was generally no significant effect based on the type (BA or topolins) and concentration of applied CKs. However, evidence of the vital influence of NAA (either alone or in combination with CKs) on morphological growth and development during micropropagation and subsequent *ex vitro* acclimatization was established. The influence of the applied PGRs on secondary metabolites and antioxidant activity of *E*. *autumnalis* subspecies *autumnalis* was highlighted. In addition, when the *in vitro* regenerants were acclimatized, there was a steady (several-fold higher) accumulation of quantified phytochemicals and antioxidant activity in the 4-month-old plants. Nevertheless, a detailed phytochemical profiling will be necessary to provide further insights on the identity of specific bioactive compounds in *E*. *autumnalis* subspecies *autumnalis*. Overall, the current findings highlight the need for an appropriate choice of PGR is as it remains critical to enhance the micropropagation of *E*. *autumnalis* subspecies *autumnalis*.

Chapter 5: Influence of smoke-water, karrikinolide and cytokinin analogues on shoot proliferation, phytochemical and antioxidant content of *in vitro* derived *Eucomis autumnalis* subspecies *autumnalis*

5. 1. Introduction

Plant productivity remains a research priority for sustaining the increasing population. In an attempt to meet the global increasing demand for plants and associated products, the vital role of plant growth regulators (PGRs) or stimulants/substances for regulating plant growth and development is well-documented **(SANTNER et al. 2009; ZALABÁK et al. 2013)**. Generally, PGRs including the naturally-occurring phytohormones, synthetic compounds and analogues modify plant growth and developmental patterns as well as exert a profound influence on many physiological processes **(GASPAR et al. 1996; JALEEL et al. 2009)**. Recently, the use of non-conventional PGRs including bio-stimulants has gained more attention and has demonstrated significant potential in propagation of several plant species **(MISRA and SRIVASTAVA 1991; GIRIDHAR et al. 2005; JALEEL et al. 2009; KULKARNI et al. 2011; AREMU et al. 2012a)**. As highlighted by these aforementioned authors, compounds such as smoke-water (SW), karrikinolide (KAR_1), triazole and triacontanol significantly improve growth and phytochemical levels in a number of plant species.

Humans have utilized smoke and fire (smoke-technology) for various agricultural purposes for centuries **(KULKARNI et al. 2011)**. Furthermore, scientific evidence on the positive role of SW on several plant species has been widely recognized **(VAN STADEN et al. 2000)**. With the isolation of the active compound **(FLEMATTI et al.**

2004; VAN STADEN et al. 2004), the field has generated great interest and witnessed an exponential growth in terms of the number of papers appearing in the literature. One of the benefits arising from the isolation of the active compound is that it has eliminated disparity and ambiguity often associated with SW **(LIGHT et al. 2009)**. While no two batches of SW contain exactly the same balance or concentration of compounds, the use of KAR_1 allows for a valid comparison of biological activities during experiments. Presently, karrikins (including karrikinolide KAR_1 and 3-methyl-2*H*-furo[2,3-c]pyran-2-one previously termed butenolide) are referred to as a new family of PGRs **(CHIWOCHA et al. 2009; DIXON et al. 2009)**. In addition to the high possibility of the interaction with other PGRs **(CHIWOCHA et al. 2009)**, SW and KAR_1 exhibited cytokinin (CK) and auxin-like activity in the mungbean bioassay **(JAIN et al. 2008)**. Both SW and KAR_1 have demonstrated potential as useful tools for enhancing plant productivity via their influence on plant growth and development but remain highly unexplored in micropropagation protocols **(LIGHT et al. 2009; KULKARNI et al. 2011)**.

Another approach to improve plant growth and development is via regulation of the metabolic pathways of PGRs. On this basis, it is generally known that CK homeostasis and signalling components have emerged as engineered targets for manipulating plant growth and development **(SANTNER and ESTELLE 2009; ZALABÁK et al. 2013; ŠMEHILOVÁ and SPÍCHAL 2014)**. For instance, modulating the CK status with inhibitors of CK perception and/or degradation may influence general plant growth and development. Based on this concept, **SPÍCHAL et al. (2009)** identified the first known molecule antagonizing the activity of the CK at the receptor level. The compound, 6-(2-hydroxy-3-methylbenzylamino)purine was

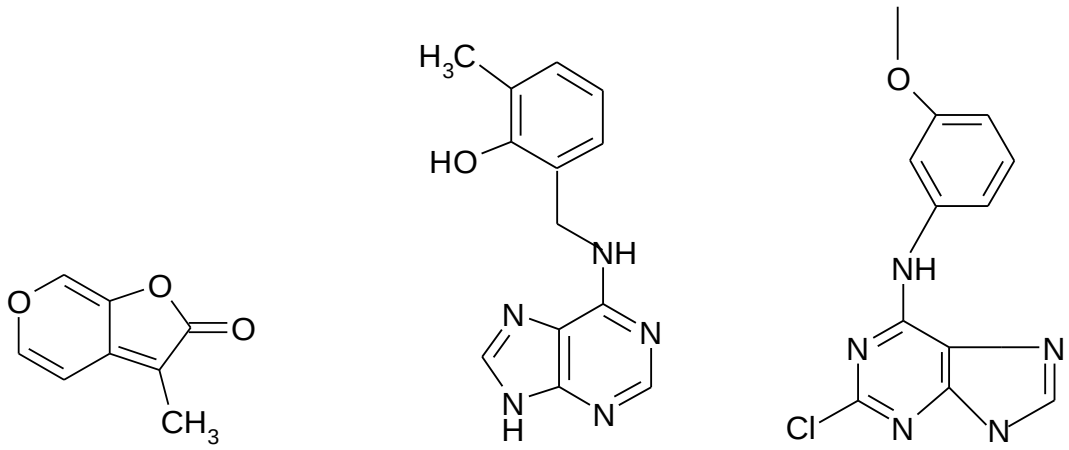
designated as PI-55 and recently tested in two medicinal plant species **(GEMROTOVÁ et al. 2013)**. The inhibition of CK oxidase/dehydrogenase also offers a target to modulate CK levels in plants. Among the compounds which have been identified as a potent inhibitor of this enzyme is 2-chloro-6-(3-methoxyphenyl)aminopurine, designated as INCYDE (inhibitor of CK oxidase/dehydrogenase) **(ZATLOUKAL et al. 2008)**. Recently, evidence on the potential of INCYDE in different aspects of plant growth and development has been documented **(AREMU et al. 2012d; GEMROTOVÁ et al. 2013; REUSCHE et al. 2013)**.

Although the application of these aforementioned compounds are steadily gaining interest by researchers, their use in micropropagation protocols especially, for valuable and highly utilized plant species **(MOYO et al. 2011)** have not been fully explored. In the current study, the influence of SW, KAR_1 and CK analogues (PI-55 and INCYDE) alone or interactions with the commonly used PGRs during micropropagation of *Eucomis autumnalis* subspecies *autumnalis* was evaluated. The value of cultivated medicinal plants is often a function of the quantity and quality of accumulated phytochemicals which inevitably determines its bioactivities **(CANTER et al. 2005)**. Thus, phytochemical levels and antioxidant activity in the regenerated *E*. *autumnalis* subspecies *autumnalis* were evaluated.

5.2. Materials and methods

5.2.1. Sources of chemicals

Smoke-water and KAR_1 **(Fig. 5.1)** were obtained from laboratory stock as described by **BAXTER et al. (1994)** and **VAN STADEN et al. (2004)**, respectively. The Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany AS CR (Olomouc, Czech Republic) provided the PI-55 and INCYDE **(Fig. 5.1)**. The compounds PI-55 and INCYDE were prepared as described by **SPÍCHAL et al. (2009)** and **ZATLOUKAL et al. (2008)**, respectively. Benzyladenine (BA) and α-naphthalene acetic acid (NAA) were purchased from Sigma-Aldrich (Steinheim, Germany).



KAR_1 PI-55 INCYDE

Fig. 5.1: Chemical structures of three compounds tested in the current study. KAR_1 = karrikinolide, PI-55 = 6-(2-hydroxy-3-methylbenzylamino)purine, INCYDE (inhibitor of cytokinin dehydrogenase) = 2-chloro-6-(3-methoxyphenyl)aminopurine.

5.2.2. Explant source *and in vitro* shoot proliferation experimental design

Aseptically-obtained leaves derived from primary bulb regenerants as in **Section 3.2.2** and subcultured on PGR-free medium were used for all experiments in the current **Chapter**. Three leaf explants, each measuring approximately 1 × 1 cm were inoculated in screw-cap jars (110 x 60 mm, 300 ml volume) each containing 30 ml Murashige and Skoog (MS) medium **(MURASHIGE and SKOOG 1962)**. The medium was supplemented with varying concentrations of the tested compounds, combined with BA, NAA or both **(Table 5.1)**. The choice of BA and NAA concentration used was based on a previous study **(TAYLOR and VAN STADEN 2001b)**. Each treatment had 15 explants and the experiments were conducted twice. The cultures were incubated in 16/8 h light/dark conditions with a photosynthetic photon flux (PPF) of 45 µmol m^{-2} s^{-1} at 25 ± 2 °C for 10 weeks. Thereafter, parameters including the shoot (number and length) and root (number and length) growth as well as plant fresh weight were measured.

Table 5.1: Summary of the different treatments used for *in vitro* shoot proliferation experiment.

Plant growth regulators	Tested compounds			
	SW (dilution)	KAR_1 (M)	PI-55 (µM)	INCYDE (µM)
PGR-free	0	0	0	0
4 µM BA	1:500	10^{-7}	0.01	0.01
5 µM NAA	1:1000	10^{-8}	0.1	0.1
4 µM BA + 5 µM NAA	1:1500	10^{-9}	10	10

BA = Benzyladenine, NAA = Naphthalene acetic acid, SW = Smoke-water, KAR_1 = Karrikinolide, PI-55 = 6-(2-hydroxy-3-methylbenzylamino)purine, INCYDE = 2-chloro-6-(3-methoxyphenyl)aminopurine

5.2.3. Phytochemical and antioxidant evaluation of *in vitro* regenerants

In vitro regenerants from the PGR-free and different treatments (SW, KAR_1, PI-55 and INCYDE) harvested after 10 weeks of culture as described above were evaluated for phytochemical content. The plant materials were oven-dried at 50 ± 2 ºC for 7 days and milled into powder form. Ground samples were extracted in 50% methanol (MeOH) at 0.1 g per 10 ml in an ultrasonic sonicator (Julabo GmbH, West Germany) containing ice-cold water for 20 min. The extracts were centrifuged and the resultant filtrate used for phytochemical quantification as outlined in **Section 3.2.4**. Iridoid, condensed tannin, flavonoid and phenolic content was expressed as mg harpagoside equivalents (HE), cyanidin chloride equivalents (CCE), catechin equivalents (CE) and gallic acid equivalents (GAE) per g dry weight (DW), respectively. Each sample had six replicates.

For antioxidant activity, ground plant materials from the different treatments were extracted as described in **Section 3.2.4**. The dried extracts were re-suspended in 50% MeOH and evaluated at a final concentration of 0.5 mg/ml in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *β*-carotene acid model system bioassays. Ascorbic acid and butylated hydroxytoluene were used as positive controls in DPPH and *β*-carotene assays respectively, while 50% MeOH was included as the solvent control. Details of DPPH and *β*-carotene antioxidant assays are described in **Section 4.2.6.1** and **4.2.6.2**, respectively. Each sample had six replicates.

5.2.4. Data analysis

Experiments were conducted in completely randomized designs. The growth, phytochemical contents and antioxidant activity data were subjected to analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 16.0 Chicago, USA). Where there was statistical significance ($P \leq 0.05$), the mean values were further separated using the Duncan's Multiple Range Test (DMRT).

5.3. Results and discussion

5.3.1. Effect of SW, KAR_1 and plant growth regulators on *in vitro* shoot and root production

The ability of SW and its derived compounds to interact with various hormones and to even mimic some hormonal activities has been recognized **(CHIWOCHA et al. 2009)**. The effect of the different dilutions of SW and KAR_1 concentrations with or without PGRs on shoot and root proliferation is presented in **Fig. 5.2**. The highest shoot number (c.a 8 shoots/explant) was observed in the treatment containing NAA alone. However, addition of either SW or KAR_1 with NAA resulted in a reduction of shoot number in *E*. *autumnalis* subspecies *autumnalis* **(Fig. 5.2A and B)**. Despite the closely interwoven relationship in function among phytohormones, evidence has shown that some developmental processes are unique to some type of phytohormones **(DEPUYDT and HARDTKE 2011)**. As shown in the current study, the decrease in shoot proliferation with the application of SW or KAR_1 has been reported during the micropropagation of 'Williams' bananas **(AREMU et al. 2012a)**. **MA et al. (2006)** also observed that KAR_1 had no significant stimulatory effect during

the earlier induction phase of somatic embryogenesis in *Baloskion tetraphyllum*. Nonetheless, positive effects of SW and KAR_1 in other aspects of micropropagation have been observed. For instance, SW and KAR_1 enhanced maturation and root formation of somatic embryos of some plant species **(SENARATNA et al. 1999; MA et al. 2006; GHAZANFARIA et al. 2012)** and improved callus biomass in the soyabean callus bioassay **(JAIN et al. 2008)**. In PGR-free regenerants, SW (1:1000) treatment significantly increased shoot length while KAR_1 (regardless of the concentration) had no positive effect when compared to the PGR-free without SW and KAR_1 **(Fig. 5.2C and D)**. Smoke-water and KAR_1 had an inhibitory effect on BA alone and BA with NAA regenerants on the number and length of the roots **(Fig. 5.2E - H)**. However, with the PGR-free regenerants, there was more roots in KAR_1 (10^{-8} M) treatment than in the control **(Fig. 5.2F)**. Similarly, the root length in regenerants derived from SW (1:1500) treatment was significantly longer than the control **(Fig. 5.2G)**.

It is known that various growth and developmental processes can be modulated by phytohormones occasionally in synergistic or antagonistic manners, an indication of a cross talk between different pathways **(DEPUYDT and HARDTKE 2011)**. Yet, it is not clear whether phytohormones target common or different transcriptome modules. Notwithstanding, both additive **(e.g. Fig. 5.2D)** and antagonistic **(e.g. Fig. 5.2A and B)** effects resulting from the interaction of the PGR with SW or KAR_1 were evident in the current study. Based on the findings using mungbean and soyabean callus bioassays, **JAIN et al. (2008)** suggested an interaction between KAR_1 and exogenous CK (kinetin) and auxin (IBA) resulting in enhanced physiological responses in both bioassays. In a similar manner, **NELSON et al. (2009)** discovered

the ability of KAR_1 to enhance germination of *Arabidopsis* seed mediated by a partial up-regulation of gibberellic acid biosynthesis.

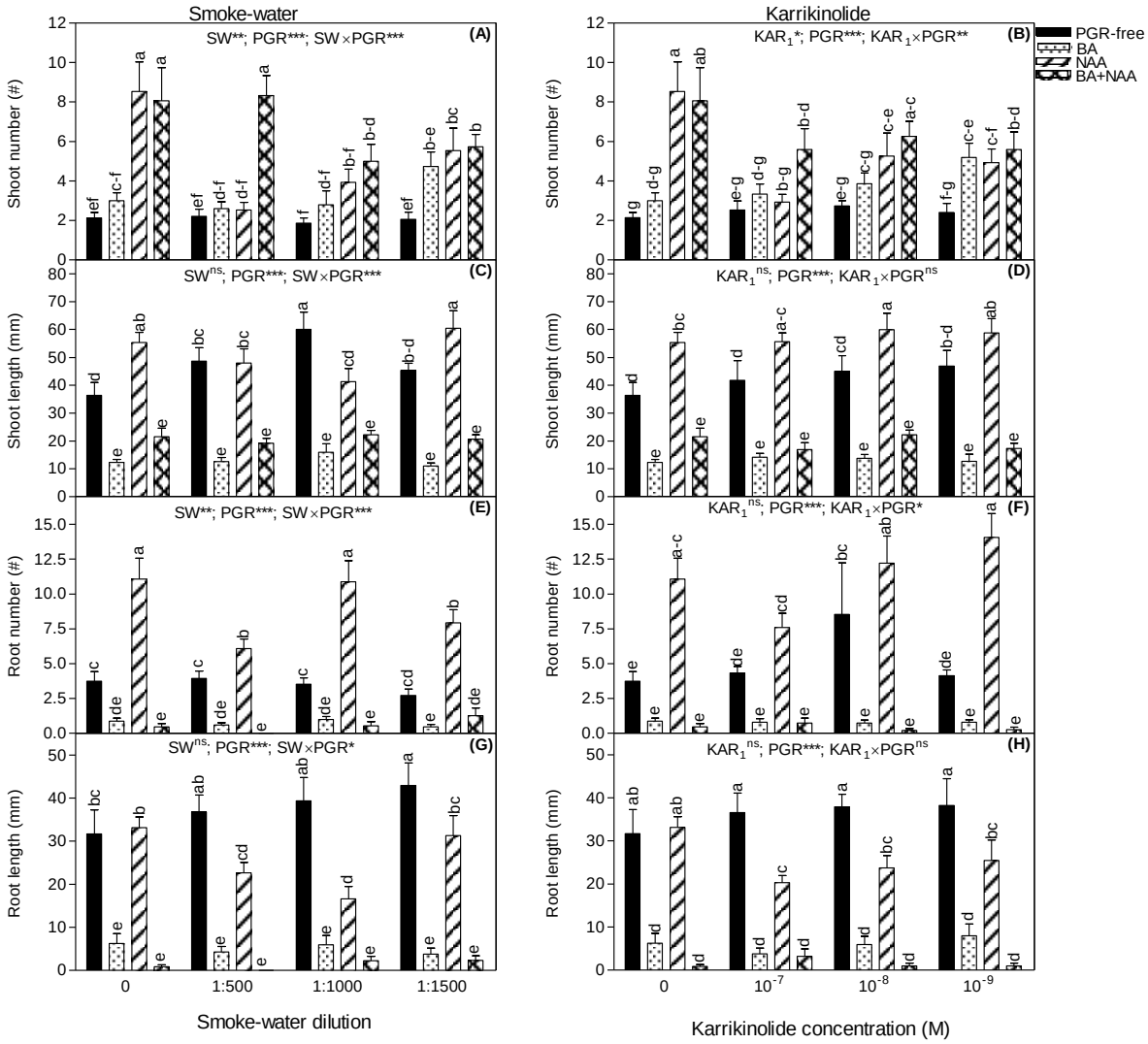


Fig. 5.2: Effect of smoke-water, karrikinolide and plant growth regulators (PGR) on (A and B) shoot number, (C and D) shoot length, (E and F) root number and (G and H) root length in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 30) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant , $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.2. Effect of PI-55 and plant growth regulators on *in vitro* shoot and root production

The concentration of PI-55, type of PGR and their interaction had a significant effect on the number of regenerated *E*. *autumnalis* subspecies *autumnalis* shoots **(Fig. 5.3A)**. Mean shoot proliferation was lowest (c.a 2 shoots/explant) in PGR-free and highest (c.a 8 shoots/explant) in 0.01 µM PI-55 with BA and NAA treatment. In the absence of PGRs, application of PI-55 (at all concentrations) had no significant stimulatory effect on the number of shoots. Structurally, PI-55 is closely related to BA, but substitutions at *meta* (CH_3) and *ortho* (OH) positions of the aromatic side chain strongly diminished its CK activity which is responsible for its antagonistic property **(SPÍCHAL et al. 2009)**. Considering that PI-55 is an inhibitor of CK activity, the non-stimulatory effect on shoot proliferation implies that a substantial level of (endogenous) CK is essential for shoot induction during micropropagation of *E*. *autumnalis* subspecies *autumnalis*. Similarly, PI-55 with the different PGR treatments produced either equal or lower numbers of shoots when compared to the treatments lacking PI-55. The longest shoots were observed in 0.1 µM PI-55 with NAA treatment while regenerants from BA with or without varying concentration of PI-55 had the shortest shoots **(Fig. 5.3B)**. On the other hand, root production in *E*. *autumnalis* subspecies *autumnalis* was significantly affected by PI-55 concentration and PGR type as well as their interaction **(Fig. 5.3C)**. At similar PI-55 concentration (with exception of 0.01 and 10 µM), the number of roots in the regenerants from medium supplemented with NAA were significantly higher than those from PGR-free, BA and BA with NAA. Root length was enhanced with varying concentrations (0.01 and 0.1 10 µM) of PI-55 in PGR-free regenerants **(Fig. 5.3D)**. As an indication of reduced CK perceptions, positive effects on rooting parameters have been demonstrated by

other researchers. For instance, CK-deficient *Arabidopsis* plants initiated more lateral root primordia, which elongated more rapidly than those of wild-type plants **(WERNER et al. 2003)**. **SPÍCHAL et al. (2009)** also showed that PI-55 accelerated the germination of *Arabidopsis* seeds and promoted root growth and formation of lateral roots. Using two important medicinal plants grown under cadmium stress, PI-55 treatment effectively stimulated root development in the seedlings **(GEMROTOVÁ et al. 2013)**. Together with other newly discovered CK antagonists such as (2,5-dihydroxybenzylamino)purine (LRG-1) **(NISLER et al. 2010)** and 6-(benzyloxymethyl)adenosine (BOMA) **(KRIVOSHEEV et al. 2012)**, PI-55 may serve as a valuable chemical for better understanding of plant response during micropropagation. In addition, these compounds may be helpful for the manipulation and regulation of micropropagation protocols as well as elucidation of the physiological basis for the *in vitro*-induced physiological disorders. A classic example is the potential application for possible induction and enhancement of *in vitro* rooting for recalcitrant species as reported for *Eucalyptus globulus* **(FOGAÇA and FETT-NETO 2005)**, *Uniola paniculata* **(VALERO-ARACAMA et al. 2010)** and *Barleria argillicola* **(AMOO and VAN STADEN 2013b)**. It has long been established that the nature and type of the exogenous CK may be responsible for root inhibition in some species **(WERBROUCK et al. 1995)**. Thus, the importance of rooting in micropropagated plants cannot be over-emphasized as it remains fundamental to *ex vitro* establishment of the regenerants.

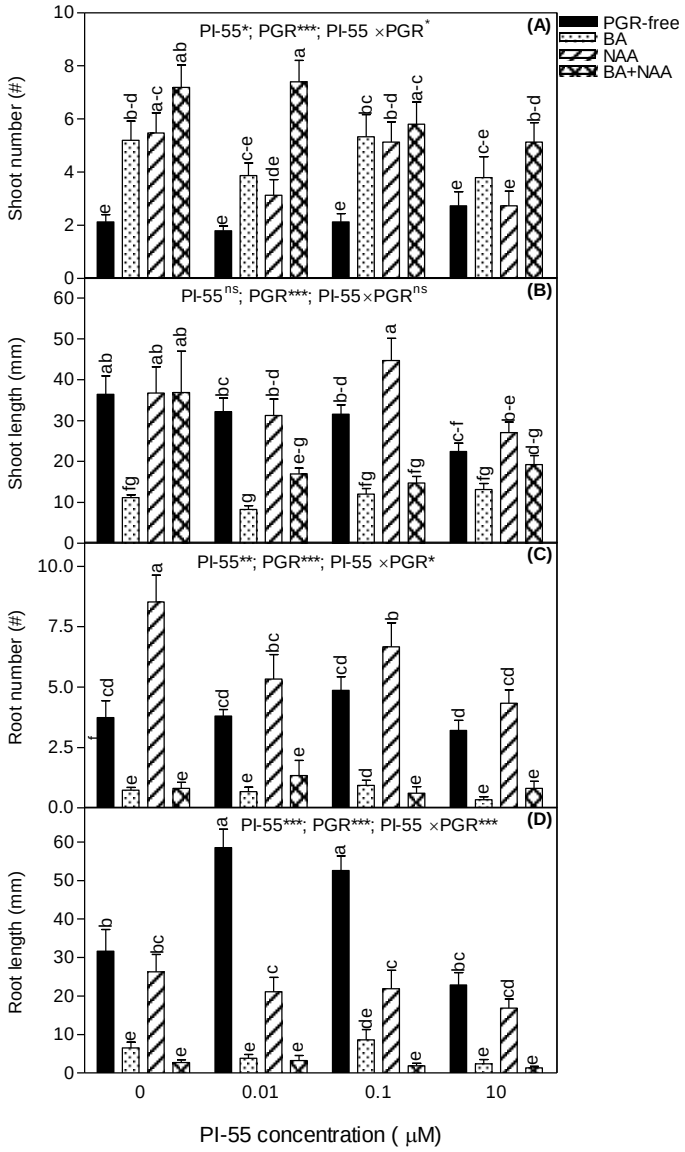


Fig. 5.3: Effect of PI-55 and plant growth regulators (PGR) on (A) shoot number, (B) shoot length, (C) root number and (D) root length in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 30) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant , $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.3. Effect of INCYDE and plant growth regulators on *in vitro* shoot and root production

The ability to alter CK metabolic pathways has been postulated to portray interesting potential and application in plant biotechnology **(ŠMEHILOVÁ and SPÍCHAL 2014)**.

As alluded by these authors, INCYDE is one of such compounds which allows for the manipulating of endogenous CK levels in plants. The potential of INCYDE stems from its ability to inhibit CK oxidase/dehydrogenase, an enzyme that is responsible for most of the CK catabolism and inactivation (mainly isoprenoid type) in a single enzymatic step **(SCHMÜLLING et al. 2003)**. Even though the different concentrations of INCYDE had no remarkable effect, the type of applied PGR and their interaction with INCYDE had a significant influence on shoot proliferation in *E*. *autumnalis* subspecies *autumnalis* **(Fig. 5.4A)**. The highest number of shoots (9 shoot/explants) was observed in a treatment containing 0.1 µM INCYDE with BA and NAA. In addition, a significant increase in number of shoots was observed in treatment containing 0.1 µM INCYDE and BA when compared to the treatment with BA alone. This observed increase in shoot number suggests the possibility of achieving additive effects at optimum concentrations of INCYDE. While their interaction had no effect, the concentration of INCYDE or type of PGR individually had a significant influence on the shoot length of the *in vitro* regenerants **(Fig. 5.4B)**. At 10 µM INCYDE, the shoot length in all the regenerants (except BA alone) was reduced when compared to those obtained from INCYDE-free treatments. *In vitro* regenerants from 0.01 µM INCYDE with NAA produced the highest number of roots **(Fig. 5.4C)**. Although there is no previous evidence on interaction between INCYDE and auxins, the current findings suggest a possible additive interaction (albeit at low concentrations of INCYDE and NAA) which stimulated increased root production in *E*. *autumnalis* subspecies *autumnalis*. In terms of root length, PGR-free treatment with 0.01 µM INCYDE produced the longest roots **(Fig. 5.4D)**. In treatments containing NAA alone with INCYDE, the regenerants had reduced root length with an increase in INCYDE concentration. Given the substantial evidence on the role of

INCYDE on endogenous CK levels **(AREMU et al. 2012d; REUSCHE et al. 2013)**, it is conceivable to attribute the observed reduction in rooting at higher INCYDE concentrations to elevated endogenous CK pools. However, it will be necessary to quantify these CKs in *E*. *autumnalis* subspecies *autumnalis* in order to reach a logical conclusion on how the influence is being exerted.

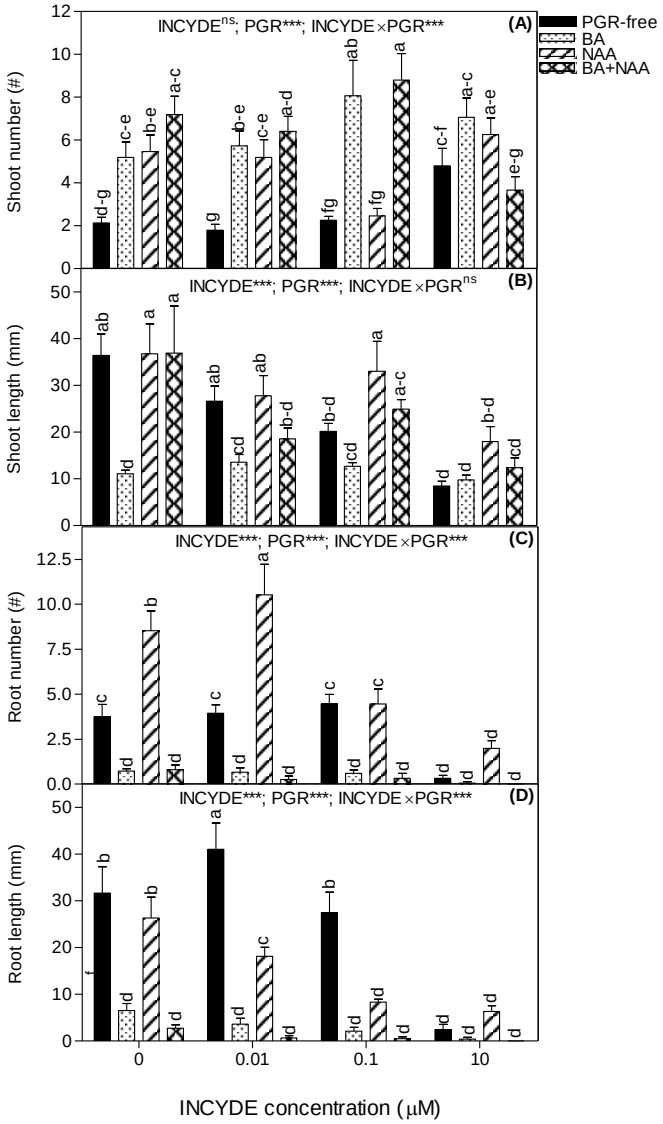


Fig. 5.4: Effect of INCYDE and plant growth regulators (PGR) on (A) shoot number, (B) shoot length, (C) root number and (D) root length in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 30) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant , $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.4. Effect of SW, KAR_1 and plant growth regulators on phytochemical content of *in vitro* regenerants

The effects of SW, KAR_1 and PGR types on phytochemical contents of *in vitro E. autumnalis subspecies autumnalis* is depicted in **Fig. 5.5**. Among plant secondary metabolites, the iridoid biosynthesis pathway is more closely related to that of alkaloids and are known to be susceptible to a number of regulatory mechanisms **(VERPOORTE et al. 2002)**. Although the use of either SW or KAR_1 alone had no significant stimulatory effect on levels of iridoid in *E. autumnalis subspecies autumnalis*, combination of SW (1:1000) or KAR_1 (10^{-7} and 10^{-8} M) with NAA treatment stimulated the highest iridoids in the regenerants **(Fig. 5.5A and B)**. A stimulatory effect of SW (with a variety of CKs) on iridoid levels was observed in *Aloe arborescens* **(AMOO et al. 2013)**. The authors suggested a potential synergistic or additive interaction of the CKs with SW on iridoid biosynthesis and accumulation in plants. While the additive effect was pronounced between auxin (NAA) and SW in the present study, it was evident that SW (1:500) with CK (BA) caused a decline in the level of iridoids in the regenerants **(Fig. 5.5A)**. The observed differences in response suggest that biosynthesis and production of iridoids in *E. autumnalis subspecies autumnalis* were differentially affected depending on the type of PGR.

When compared to treatments lacking SW, BA or BA with NAA in combination with SW (1:500 and 1:1500) induced a significantly higher level of condensed tannins in the regenerated *E. autumnalis* subspecies *autumnalis* SW **(Fig. 5.5C)**. Conversely, regenerants from BA with various dilutions of SW had remarkable lower condensed tannins than the treatment with BA alone. About 8-fold higher condensed tannins was accumulated in 10^{-7} M KAR_1 (BA with NAA treatment) when compared to the

control without KAR_1 **(Fig. 5.5D)**. A similar stimulatory effect was observed with PGR-free regenerants cultured on media supplemented with 10^{-7} and 10^{-9} M KAR_1. Unlike BA alone, KAR_1 at all concentrations with BA caused a significant reduction in concentration of condensed tannin in the regenerants. Among the diverse secondary metabolites, the therapeutic value of condensed tannins cannot be over-emphasized **(XIE and DIXON 2005)**. The phenomenal increase in condensed tannins may inevitably enhance the biological activity of the regenerants.

The highest flavonoid content was observed in PGR-free regenerants containing SW (1:1000) **(Fig. 5.5E)**. Among the tested PGRs, BA or NAA treatments accumulated higher levels of flavonoids in the presence of SW (1:500 and 1:1500) than the SW-free regenerants. Relative to the PGR-free *E. autumnalis subspecies autumnalis* regenerants, an approximately 3-fold increase in flavonoid content was recorded with 10^{-7} M KAR_1 (either alone or when combined with BA and NAA) **(Fig. 5.5F)**. Among the PGR-free treatments, the addition of SW (all dilutions) and KAR_1 (all concentrations) significantly improved the phenolic content in *E. autumnalis subspecies autumnalis* compared to treatments lacking SW or KAR_1 **(Fig. 5.5G and H)**. *Eucomis autumnalis* subspecies *autumnalis* obtained from media containing SW with BA (1:500) or NAA (1:1500) had higher phenolic content than regenerants from BA or NAA alone **(Fig. 5.5G)**. Likewise, there was a higher phenolic content with KAR_1 at 10^{-7} M (BA and NAA) or 10^{-8} M (NAA) when compared to regenerants with similar PGR without KAR_1 **(Fig. 5.5H)**. Recently, there has been an increase in the number of studies demonstrating the stimulatory role of SW and KAR_1 on phytochemical levels in plants both under *in vitro* **(AREMU et al. 2012a; 2014)** and *ex vitro* **(ZHOU et al. 2011; KULKARNI et al. 2013)** conditions. Based on molecular

evidence, the stimulatory effects of SW and KAR_1 have been attributed to the modulation of the phenylpropanoid pathway and up-regulation of flavonoid-related genes **(SOÓS et al. 2010)**.

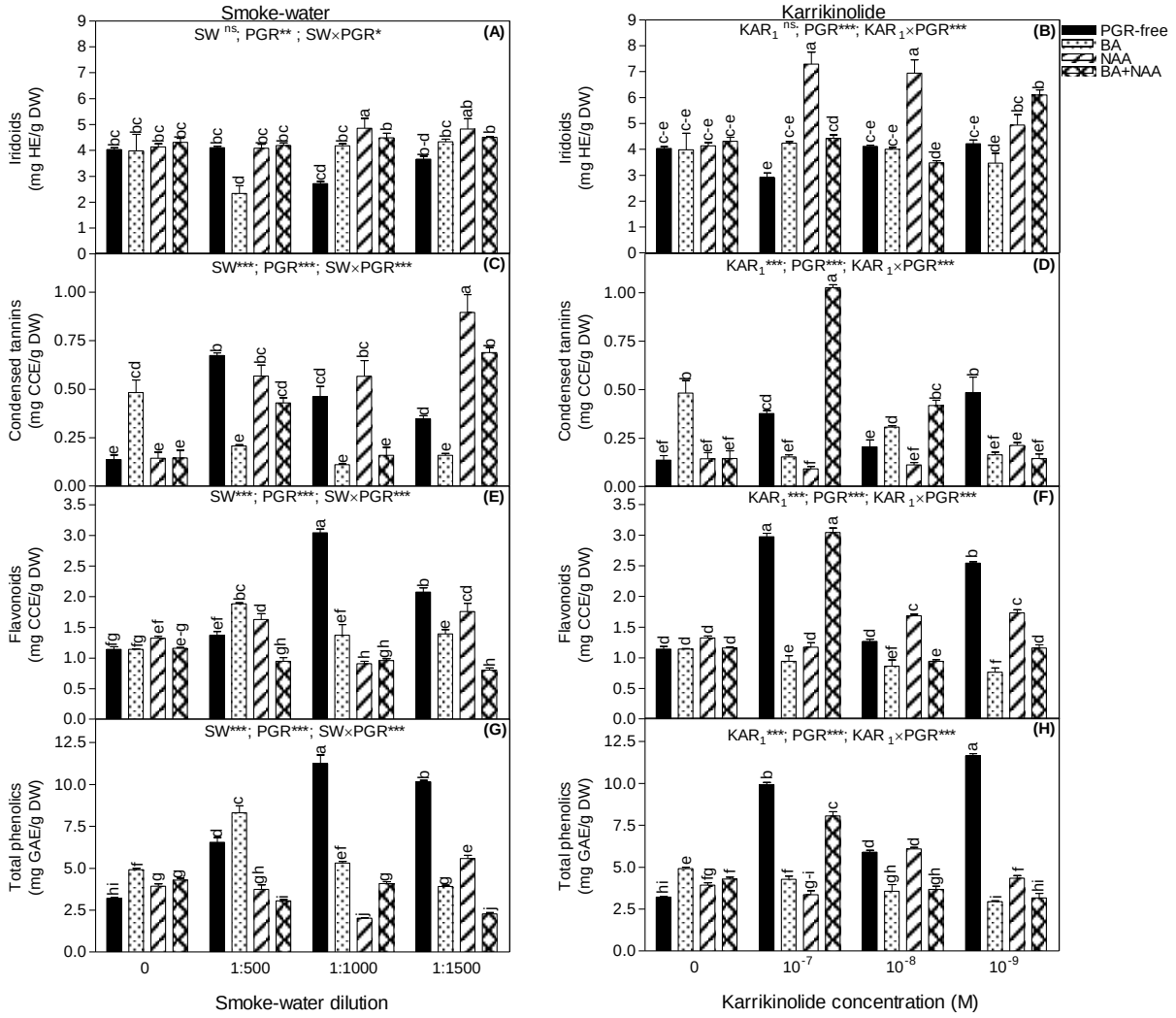


Fig. 5.5: Effect of smoke-water, karrikinolide and plant growth regulators (PGR) on (A and B) Iridoids, (C and D) condensed tannins, (E and F) flavonoids and (G and H) phenolics in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 6) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant , $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.5. Effect of PI-55 and plant growth regulators on phytochemical content of *in vitro* regenerants

Based on the vital role of CKs on the growth cycle, the presence of CK analogues could influence the production of essential plant secondary metabolites **(PLANCHAIS et al. 2000)**. Overall, PI-55 concentrations, PGRs and their interaction significantly influenced the quantified phytochemical content in *E*. *autumnalis* subspecies *autumnalis* **(Fig. 5.6)**. In PGR-free medium, regenerants with 0.1 and 10 µM PI-55 had significantly higher iridoid content than the lower (0 and 0.01 µM) concentrations **(Fig 5.6A)**. In contrast, BA (with 10 µM PI-55) and NAA (with 0.01 and 0.1 µM PI-55) treatments had lower concentrations of iridoids than the similar PGR treatments lacking PI-55. With respect to the PGR-free treatments, the 2-fold increase in iridoid content with regenerants from 0.1 µM PI-55 media when compared to media without PI-55 may offer a potential useful elicitor for iridoids in micropropagated species. As shown in **Fig 5.6B**, PGR-treated (NAA and BA + NAA) *E*. *autumnalis* subspecies *autumnalis* with 10 µM PI-55 and without PI-55 treatments had the highest condensed tannin content. Among the PGR-free regenerants, 0.1 µM PI-55 treatments yielded higher flavonoid and phenolic contents than other concentration of PI-55 (**Fig 5.6C and D)**. Furthermore, a significant increase in flavonoid and phenolic content with an increase in PI-55 concentration was observed in BA-derived regenerants. While the influence of PGRs such as NAA and BA on secondary metabolite production in micropropagated plants is common **(RAMACHANDRA RAO and RAVISHANKAR 2002; KARUPPUSAMY 2009)**, the current findings provide an indication on the significant influence of PI-55 alone and its interaction with PGRs on phytochemical levels. This hypothesis is based on evidence that exogenous BA induced the expression of phenylalanine ammonia-

lyase (key enzyme in cinnamate biosynthesis and accumulation of anthocyanins) in *Arabidopsis thalina* **(DEIKMAN and HAMMER 1995)**.

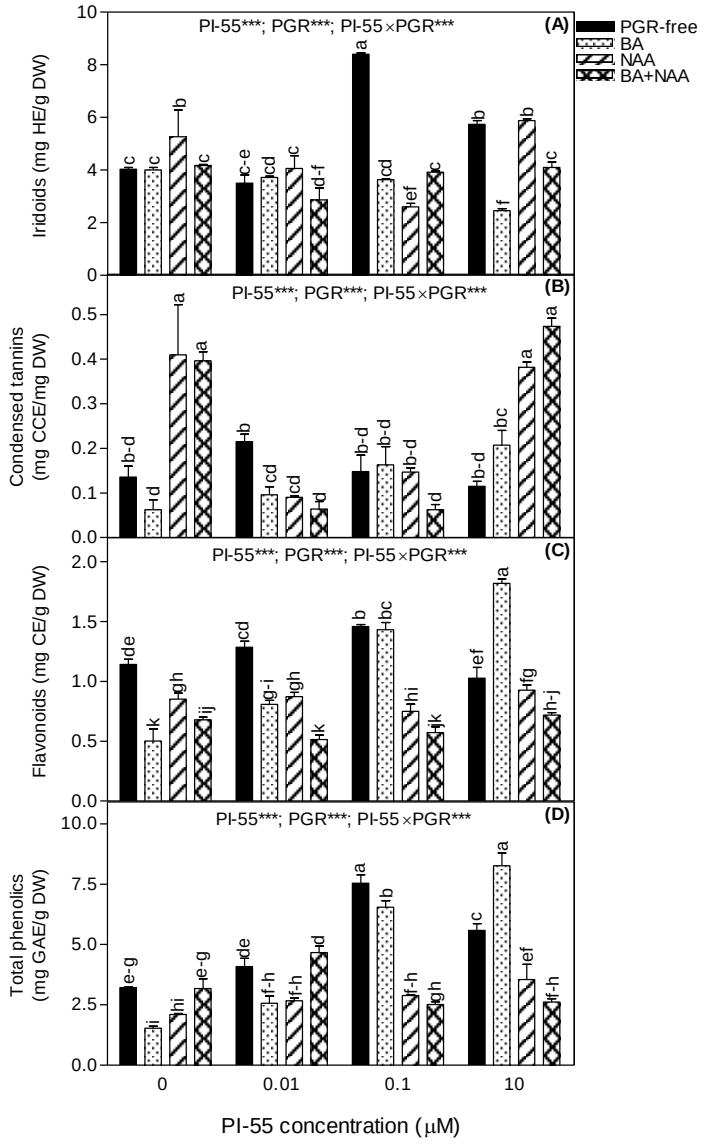


Fig. 5.6: Effect of PI-55 and plant growth regulators (PGR) on (A) Iridoids, (B) condensed tannins, (C) flavonoids and (D) phenolics in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 6) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant *,* $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.6. Effect of INCYDE and plant growth regulators on phytochemical content of *in vitro* regenerants

Apart from iridoid content which was not affected by INCYDE concentration and PGR types, the condensed tannins, flavonoids and phenolics were significantly influenced **(Fig. 5.7)**. In addition, the interaction of INCYDE and PGRs influence the levels of all the four types of phytochemicals quantified in *E*. *autumnalis* subspecies *autumnalis*. While iridoid content was reduced by 4-fold in the presence of 0.01 µM INCYDE with NAA, the same concentration of INCYDE significantly increased iridoid content in the PGR-free regenerants when compared to media without INCYDE and PGR-free **(Fig. 5.7A)**. Application of higher levels of INCYDE (0.1 and 10 µM) reduced the level of condensed tannins in PGR-treated (BA regenerants) compared to 0.01 µM INCYDE treatment **(Fig. 5.7B)**. On the other hand, PGR-free treatment with 0.01 µM INCYDE had more condensed tannins than similar treatments with or without INCYDE **(Fig. 5.7B)**. The interaction of BA or *m*T with a high concentration (100 µM) of INCYDE significantly reduced the level of condensed tannins in micropropagated *Musa* species **(AREMU et al. 2012d)**. It is possible that higher concentrations of INCYDE exert inhibitory effects on the production of condensed tannins in micropropagated plants. Regenerants from PGR-free with 0.1 µM INCYDE treatment had the most abundant (c.a 2.5 mg CE/g DW) flavonoid content **(Fig. 5.7C)**. When PGRs were added to the media, both BA and NAA treatments with 0.01 µM INCYDE were superior to other concentrations of INCYDE with BA or NAA. Among the INCYDE concentrations tested (0 - 10 µM), the highest levels of phenolics were recorded at 0.01 µM INCYDE with or without PGRs **(Fig 5.7D)**. While the therapeutic values of these quantified phytochemicals are well-recognized

(VERPOORTE et al. 2002; MATKOWSKI 2008), new approaches to increase their concentration in plants are also desirable. On this basis, the enhanced flavonoid and phenolic content in *E*. *autumnalis* subspecies *autumnalis* mediated with the use of 0.01 µM INCYDE is noteworthy. Even though the underlying mechanism of INCYDE regulating plant secondary metabolic biosynthesis pathway is yet to be elucidated, the influence may be related to its modulatory effect on CK homeostasis **(ŠMEHILOVÁ and SPÍCHAL 2014)**. Even though the molecular basis is not fully understood, it is widely acknowledged that CKs are often directly or indirectly involved in plant response to different types of stress **(ZALABÁK et al. 2013)**. Based on this hypothesis, stabilization of CK levels due to treatment with INCYDE confers enhanced resistance to the pathogen *Verticillium longisporum* in *Arabidopsis* species **(REUSCHE et al. 2013)**. Although there is no doubt that the accumulation of secondary metabolites in plants is part of the defense mechanism against pathogenic attack **(DIXON and PAIVA 1995)**, the relationship of CK as well as INCYDE in the secondary metabolite biosynthesis pathway need further studies in order to decipher the complex interactions.

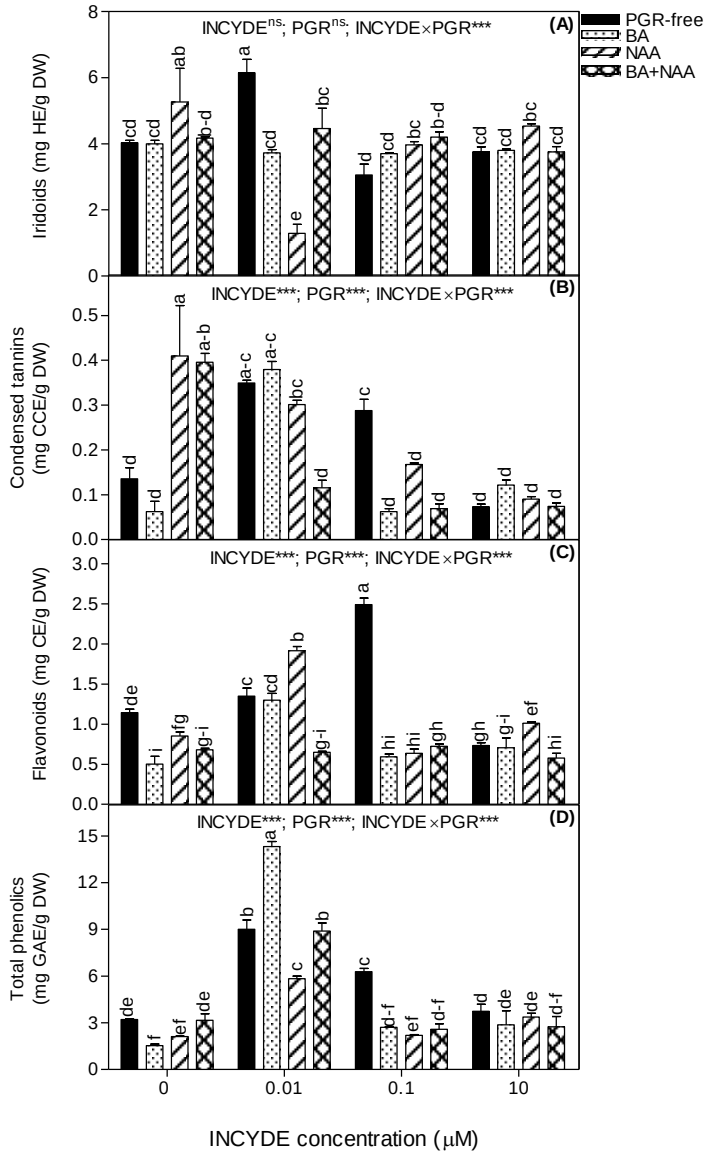


Fig. 5.7: Effect of INCYDE and plant growth regulators (PGR) on (A) Iridoids, (B) condensed tannins, (C) flavonoids and (D) phenolics in micropropagated *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture. Bars represent mean value ± standard error (n = 6) and bars with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = Benzyladenine; NAA = Naphthalene acetic acid; NS = Not significant *,* $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5.3.7. Effect of SW, KAR_1 and plant growth regulators on antioxidant activity of *in vitro* regenerants

Table 5.2 shows the effects of SW, KAR_1 and PGR types on antioxidant activity of *in vitro* regenerated *E*. *autumnalis* subspecies *autumnalis*. Smoke-water (1:1000 and

1:1500) with NAA, as well as BA and NAA (1:1000) gave significantly higher antioxidant (*β*-carotene assay) activity than either their respective control (SW-free) treatments. *Eucomis autumnalis* subspecies *autumnalis* derived from PGR-free media with KAR_1 at 10^{-9} and 10^{-7} M had the highest antioxidant activity in the DPPH and *β*-carotene assays, respectively. Despite the numerous *in vitro* approaches that have been used to enhance the biosynthesis and accumulation of antioxidant compounds in plant cells, the use of SW and KAR_1 have rarely been documented. Evidence of the vital role of PGRs on antioxidant potential in *in vitro* regenerants has been extensively reviewed **(RAMACHANDRA RAO and RAVISHANKAR 2002; MATKOWSKI 2008)**. Furthermore, **AREMU et al. (2013)** reported that some specific phenolic acids (e.g. caffeic acid, protocatechuic acid, *p*-coumaric acid) known for their antioxidant potential were several fold higher in *Merwilla plumbea* regenerated from CK supplemented media compared to the control. The current findings provide a clear indication that SW and KAR_1 have the ability to improve antioxidant activity in medicinal plants. Particularly, the use of SW affords a cheaper alternative to enhancing the antioxidant activity (and possibly other pharmacological activities) in medicinal species.

Table 5.2: Effect of smoke-water, karrikinolide and plant growth regulators on the antioxidant activity of *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Treatment		Antioxidant activity (%) of SW regenerants				Antioxidant activity (%) of KAR_1 regenerants					
PGR	SW dilution	DPPH		*Beta*-carotene		PGR	KAR_1 conc. (M)	DPPH		*Beta*-carotene	
PGR-free	0	26.3 ± 2.48	d-g	41.9 ± 3.84	g	PGR-free	0	26.3 ± 2.48	fg	41.9 ± 3.84	f
	SW 1:500	34.0 ± 3.91	b-d	58.3 ± 1.68	ef		10^{-7}	46.9 ± 4.36	b	97.5 ± 1.02	a
	SW 1:1000	54.8 ± 0.62	a	44.5 ± 5.34	g		10^{-8}	37.9 ± 1.05	de	35.0 ± 4.28	f
	SW 1:1500	50.5 ± 7.00	a	43.9 ± 1.85	g		10^{-9}	55.1 ± 1.14	a	92.9 ± 3.60	ab
BA	0	19.6 ± 1.07	fg	93.9 ± 2.55	ab	BA	0	19.6 ± 1.07	hi	93.9 ± 2.55	ab
	SW 1:500	18.2 ± 1.37	g	60.4 ± 4.19	ef		10^{-7}	28.2 ± 4.68	f	93.3 ± 4.79	ab
	SW 1:1000	27.8 ± 1.61	d-f	43.8 ± 1.01	g		10^{-8}	23.2 ± 0.20	f-h	82.8 ± 4.90	bc
	SW 1:1500	27.0 ± 1.64	d-f	48.1 ± 3.81	fg		10^{-9}	44.5 ± 2.58	b-d	91.9 ± 0.81	ab
NAA	0	36.2 ± 2.41	bc	67.9 ± 6.35	de	NAA	0	36.2 ± 2.41	e	67.9 ± 6.35	de
	SW 1:500	57.0 ± 1.04	a	76.9 ± 6.33	cd		10^{-7}	45.8 ± 1.91	bc	62.3 ± 1.14	e
	SW 1:1000	41.6 ± 0.18	b	85.9 ± 3.17	a-c		10^{-8}	23.3 ± 0.36	f-h	41.5 ± 0.45	f
	SW 1:1500	52.0 ± 3.17	a	83.8 ± 4.73	bc		10^{-9}	39.5 ± 0.41	c-e	63.9 ± 4.18	de
BA+NAA	0	22.7 ± 1.79	e-g	61.4 ± 5.21	e	BA+NAA	0	22.7 ± 1.79	f-h	61.4 ± 5.21	e
	SW 1:500	32.4 ± 2.35	cd	45.2 ± 3.92	g		10^{-7}	36.1 ± 2.13	e	87.6 ± 0.89	a-c
	SW 1:1000	28.5 ± 1.53	c-e	96.9 ± 1.96	a		10^{-8}	17.6 ± 0.88	hi	82.0 ± 4.22	bc
	SW 1:1500	23.0 ± 0.76	e-g	67.3 ± 5.35	de		10^{-9}	13.5 ± 1.05	i	75.7 ± 6.45	cd
SW		$P < 0.001$		$P = 0.029$		KAR_1		$P < 0.001$		$P < 0.001$	
PGR		$P < 0.001$		$P < 0.001$		PGR		$P < 0.001$		$P < 0.001$	
SW × PGR		$P < 0.001$		$P < 0.001$		KAR_1 × PGR		$P < 0.001$		$P < 0.001$	

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). DPPH = 2,2-diphenyl-1-picrylhydrazyl; BA = 6-Benzyladenine; NAA = Naphthalene acetic acid; SW = smoke-water; KAR_1 = karrikinolide; PGR = plant growth regulator. All extracts and positive controls were evaluated at final concentration of 0.5 mg/ml. Positive controls were ascorbic acid for DPPH (97.6 ± 1.88%) and butylated hydroxytoluene for *beta*-carotene (98.8 ± 0.98%) assays.

5.3.8. Effect of PI-55 and plant growth regulators on antioxidant activity of *in vitro* regenerants

The antioxidant activity of the regenerants from the PI-55 and PGR treatments is shown in **Table 5.3**. Although PGR-free treatment with 0.1 µM PI-55 had the highest antioxidant activity in both test systems used, the *β*-carotene antioxidant activity was higher than DPPH free radical scavenging power. Similarly, *β*-carotene antioxidant activity was higher than the DPPH scavenging powers for all the treatments. As reviewed by **HUANG et al. (2005)**, the well-established differences in the mechanism of action in the two assays possibly account for the variations in antioxidant activity. In the DPPH assay, BA with 10 µM PI-55 treatment increased the antioxidant activity in the regenerants while lower concentrations of PI-55 had no positive effect. Conversely, NAA with 0.01 µM PI-55 had better antioxidant activity than the higher concentrations of PI-55. Regenerants cultured on 10 µM PI-55 with or without PGR had a lower *β*-carotene antioxidant activity when compared to other concentrations of PI-55. Presumably, the application of PI-55 at 10 µM was inhibitory to production of antioxidant compounds in *E*. *autumnalis* subspecies *autumnalis* for this test only. Although flavonoids and phenolics are often associated with antioxidant activity **(GÜLÇIN 2012)**, there was direct relationship between the phytochemical content and antioxidant activity in the micropropagated *E*. *autumnalis* subspecies *autumnalis*. Such absence of or poor direct relationship between phenolics and antioxidant capacity in different plant species has been reported by other researchers **(WOJDYŁO et al. 2007; SABEENA FARVIN and JACOBSEN 2013; AMOO et al. 2014)** and often attributed to the quality of the

phytochemicals detected. Furthermore, antioxidant activity of phenolics depends on the structure and substitution pattern of hydroxyl groups **(GÜLÇIN 2012)**.

Table 5.3: Effect of PI-55 and plant growth regulators on the antioxidant activity of *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Treatment		Antioxidant activity (%)			
PGR	PI-55 conc. (µM)	DPPH		*Beta*-carotene	
Control	0	26.3 ± 2.48	cd	68.5 ± 2.10	c-e
	0.01	23.8 ± 1.48	c-e	83.8 ± 4.73	ab
	0.1	43.4 ± 3.10	a	93.6 ± 0.56	a
	10	36.2 ± 0.72	b	64.7 ± 2.90	de
BA	0	15.7 ± 1.70	f	84.4 ± 6.69	ab
	0.01	15.5 ± 0.76	f	92.6 ± 3.58	a
	0.1	14.6 ± 0.97	f	88.6 ± 0.29	a
	10	23.5 ± 0.35	c-e	82.0 ± 5.29	a-c
NAA	0	24.8 ± 1.23	c-e	57.4 ± 6.63	d-f
	0.01	27.7 ± 3.22	cd	71.6 ± 0.86	b-d
	0.1	18.4 ± 0.47	ef	81.6 ± 7.10	a-c
	10	20.4 ± 0.38	d-f	44.9 ± 3.89	f
BA+NAA	0	27.9 ± 2.44	c	56.7 ± 1.69	ef
	0.01	16.0 ± 2.97	f	80.3 ± 7.65	a-c
	0.1	35.7 ± 4.80	b	59.2 ± 1.29	de
	10	26.8 ± 2.79	cd	44.4 ± 5.66	f
PI-55		$P < 0.001$		$P < 0.001$	
PGR		$P < 0.001$		$P < 0.001$	
PI-55 × PGR		$P < 0.001$		$P < 0.001$	

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's multiple range test (DMRT). BA = 6-Benzyladenine; NAA = Naphthalene acetic acid; PI-55 = 6-(2-hydroxy-3-methylbenzylamino)purine; PGR = plant growth regulator. All extracts and positive controls were evaluated at final concentration of 0.5 mg/ml. Positive controls were ascorbic acid for DPPH (97.6 ± 1.88%) and butylated hydroxytoluene for *beta*-carotene (98.8 ± 0.98%) assays.

5.3.9. Effect of INCYDE and plant growth regulators on antioxidant activity of *in vitro* regenerants

The effect of INCYDE concentrations and PGR types on antioxidant activity of *in vitro* regenerants is presented in **Table 5.4**. The highest antioxidant activity was recorded in PGR-free treatment supplemented with 0.01 and 10 µM INCYDE in DPPH and *β*-

carotene antioxidant assays, respectively. Considering that INCYDE is a relatively new compound, there are limited studies demonstrating its effect on antioxidant activity in plants. While there is no direct evidence on the influence of INCYDE in micropropagated plants, the ability of INCYDE to significantly improve the antioxidant activity in field-grown lettuce was reported by **GRUZ and SPICHAL (2011)**. In addition to the fact that INCYDE influences the antioxidant activity in the *E*. *autumnalis* subspecies *autumnalis* regenerants, there was a remarkable additive effect resulting from INCYDE and BA treatment than BA alone in the DPPH assay **(Table 5.4)**. On the other hand, reduced antioxidant activity was observed when INCYDE was combined with NAA as well as BA and NAA treatments at all concentrations of INCYDE when compared to these PGR treatments lacking INCYDE. Studies demonstrating the vital role of exogenously supplied PGR on resultant bioactivities in *in vitro*-derived regenerants are well documented **(UCHENDU et al. 2011; AMOO et al. 2012; 2014; BASKARAN et al. 2014)**. In the PGR-free treatment, regenerants had a lower antioxidant activity with increasing INCYDE concentrations in the DPPH test system while an increased antioxidant activity was observed in *β*-carotene antioxidant assay. Thus, INCYDE has the potential to influence the antioxidant activity of *E*. *autumnalis* subspecies *autumnalis* plants regardless of the antioxidant test systems. Given that antioxidants are known to exert antimicrobial, anti-inflammatory, anti-aging and health-promoting effects on the human body **(BECKER et al. 2014)**, it is possible that INCYDE treatment may also influence other biological activities of *E*. *autumnalis* subspecies *autumnalis*.

Table 5.4: Effect of INCYDE and plant growth regulators on the antioxidant activity of *Eucomis autumnalis* subspecies *autumnalis* after 10 weeks of culture.

Treatment		Antioxidant activity (%)			
PGR	INCYDE conc. (µM)	DPPH		*Beta*-carotene	
Control	0	26.3 ± 2.48	b-d	68.5 ± 2.10	de
	0.01	43.9 ± 2.18	a	68.4 ± 7.86	de
	0.1	30.0 ± 0.46	b	88.9 ± 2.89	a
	10	17.4 ± 2.34	f-h	94.9 ± 4.08	a
BA	0	15.7 ± 1.70	gh	84.4 ± 6.69	a-c
	0.01	31.3 ± 0.69	b	85.7 ± 0.98	ab
	0.1	20.4 ± 2.04	e-g	67.2 ± 7.52	d-f
	10	22.3 ± 2.22	d-f	84.5 ± 1.41	a-c
NAA	0	24.8 ± 1.23	c-e	57.4 ± 6.63	d-f
	0.01	16.6 ± 0.61	gh	70.8 ± 3.72	c-e
	0.1	17.5 ± 0.50	f-h	52.8 ± 1.30	f
	10	14.7 ± 1.22	h	61.9 ± 4.13	d-f
BA+NAA	0	27.9 ± 2.44	bc	56.7 ± 1.69	ef
	0.01	23.1 ± 0.14	c-e	71.5 ± 2.41	b-e
	0.1	15.7 ± 1.03	gh	38.1 ± 3.96	g
	10	14.7 ± 0.79	h	72.3 ± 6.26	b-d
INCYDE		$P < 0.001$		$P < 0.001$	
PGR		$P < 0.001$		$P < 0.001$	
INCYDE × PGR		$P < 0.001$		$P < 0.001$	

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P \leq 0.05$) based on Duncan's Multiple Range Test (DMRT). BA = 6-Benzyladenine; NAA = α-Naphthalene acetic acid; INCYDE = 2-chloro-6-(3-methoxyphenyl)aminopurine; PGR = plant growth regulator. All extracts and positive controls were evaluated at final concentration of 0.5 mg/ml. Positive controls were ascorbic acid for DPPH (97.6 ± 1.88%) and butylated hydroxytoluene for *beta*-carotene (98.8 ± 0.98%) assays.

5.4. Concluding remarks

The current findings explored the potential of SW, KAR_1 and two CK analogues (PI-55 and INCYDE) on growth, phytochemical and antioxidant activity in micropropagated *E. autumnalis* subspecies *autumnalis*. While these compounds had no significant stimulatory effect on shoot proliferation, they influenced various parameters (root number and length) at varying concentrations when applied alone and combined with applied PGRs. The current evidence indicates the array of potential processes

influenced by SW and KAR_1 in micropropagation protocols. Thus, more studies such as quantification of endogenous PGR and identification of specific phytochemicals responsible for the antioxidant activity in this species will provide better insights on the mechanism of action for both CK analogues. Overall, these findings show the potential practical use of the tested compounds in the quest to conserve and exploit valuable medicinal species.

Chapter 6: General conclusions

Eucomis autumnalis subspecies *autumnalis* is a widely used species in African Traditional Medicine (ATM) and scientific evidence to support some of the traditional claims have been documented. In view of the increasing strains on the wild populations, an existing micropropagation protocol was optimized by examining the role of physical (gelling agent) and chemical factors (e.g PGR = plant growth regulator) that influence the growth, phytochemical content and antioxidant activity of *E*. *autumnalis* subspecies *autumnalis*. Regenerants from gelrite solidified-medium had a higher shoot proliferation than agar solidified-medium. The highest shoot proliferation was approximately 9 shoots per explant. Regardless of the source of initial/primary explant source (LDL = leaf explant derived from primary leaf regenerants and LDB = leaf explant derived from primary bulb regenerants), shoot proliferation from PGR-free media were similar.

The low shoot proliferation observed with some of the tested cytokinins (CKs) was significantly improved with the addition of α-naphthalene acetic acid (NAA). In addition, evidence of the critical influence of NAA (either alone or in combination with tested CKs) on morphological growth and development during micropropagation and subsequent *ex vitro* acclimatization was established. Regenerated plants treated with NAA, *m*T (*meta*-topolin) and *m*TTHP [*meta*-topolin tetrahydropyran-2-yl or 6-(3-hydroxybenzylamino)-9-tetrahydropyran-2-ylpurine] showed improved *ex vitro* growth due to the carry-over effect attained from *in vitro* micropropagation. Even though rooting was enhanced in some cases, the application of smoke-water (SW), karrikinolide (KAR_1), PI-55, and INCYDE had minimal positive effects on shoot proliferation.

The significant influence of the applied plant growth regulators (PGRs) on phytochemical levels and antioxidant activity of *E*. *autumnalis* subspecies *autumnalis* was noticeable in the current study. There was no distinct direct relationship between the type of applied PGR and resultant phytochemicals quantified or antioxidant activity of the regenerants. Perhaps, the phytochemicals of interest will determine the choice of PGRs. When the *in vitro* regenerants were acclimatized, there was a gradual (several-fold higher) accumulation of quantified phytochemicals and antioxidant activity in the 4-month-old plants. As an indication of their regulatory role on secondary metabolite biosynthesis pathways, SW, KAR_1, PI-55, and INCYDE demonstrated a strong influence on phytochemical content such as flavonoids and phenolics in micropropagated *E*. *autumnalis* subspecies *autumnalis*. Inevitably, the antixodant activity of the regenerants was enhanced with the use of the PGRs, CK analogues, SW and KAR_1. On the basis of differences in the antioxidant reaction mechanism, the current findings show that antioxidant principle(s) in *E*. *autumnalis* subspecies *autumnalis* is/are more potent in *β*-carotene (hydrogen atom transer mechanism) than in the DPPH free radical scavenging assay (electron transfer mechanism). Based on the preliminarly nature of the phytochemical assays used in the current study, a detailed phytochemical profiling will be necessary to provide further insights on the identity of specific bioactive compounds in *E*. *autumnalis* subspecies *autumnalis*.

Apart from unravelling the potential application of SW, KAR_1, PI-55 and INCYDE in micropropagation protocols, the current findings emphasized the need for an appropriate choice of PGR as it remains critical to enhance the micropropagation of *E*.

autumnalis subspecies *autumnalis*. Considering the effectiveness of NAA for shoot proliferation in the micropropagation of this species, an endogenous phytohormone profile is pertinent as this will possibly provide a basis for the exceptional (shoot proliferation) response observed with the use of an auxin. Furthermore, it was clearly established that the effect of the PGRs are long-lasting as they not only influence the *ex vitro* growth but affect the phytochemical levels and antioxidant activity. Taken together, an improved protocol which incorporated the importance of the levels of phytochemical and antioxidant activity in *E*. *autumnalis* subspecies *autumnalis* was developed. As a conservation strategy, it is possible to adapt the current findings to other valuable medicinal species within the *Eucomis* genus with minimal effort.

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APPENDIX 1: Protocol for Murashige and Skoog basal medium

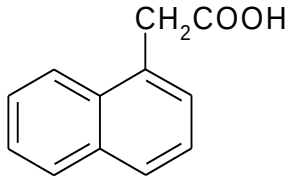
Component of stock solution

Stock	Salt component	Mass/500 ml stock (g)	Mass/1000 ml	Volume stock (ml/l) final medium
1	NH_3NO_3	82.5	165.0	10
2	KNO_3	47.5	95.0	20
3	$CaCl_2.2H_2O$	22.0	44.0	10
4	$MgSO_4.7H_2O$	18.5	37.0	10
5	NaFeEDTA	2.0	4.0	10
6	KH_2PO_4	8.5	17.0	10
7a	H_3BO_4	0.31	0.62	10
	$ZnSO_4.7H_2O$	0.430	0.860	10
	KI	0.0415	0.083	10
7b	$MnSO_4.4H_2O$	1.115	2.230	10
8	$NaMoO_4.2H_2O$	0.0125	0.025	10
	$CuSO_4.5H_2O$	0.00125	0.0025	10
	$CoCl_2.6H_2O$	0.00125	0.0025	10
9	Thiamin HCl (B1/Aneurine)	0.005	0.01	10
	Niacine (Nicotinic acid)	0.025	0.05	10
	Pyridoxine HCl (B_6)	0.025	0.05	10
	Glycine	0.1	0.2	10

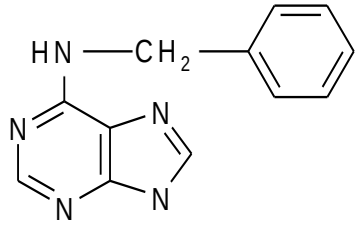
Other additives

Sugar: 30 g/l
Gelrite: 3 g/l

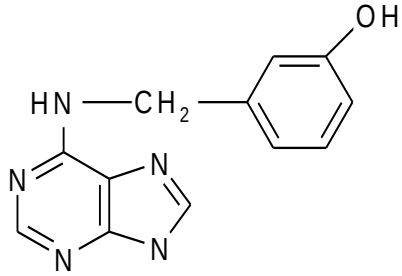
APPENDIX 2: Chemical structures of auxin and cytokinins used in the current study



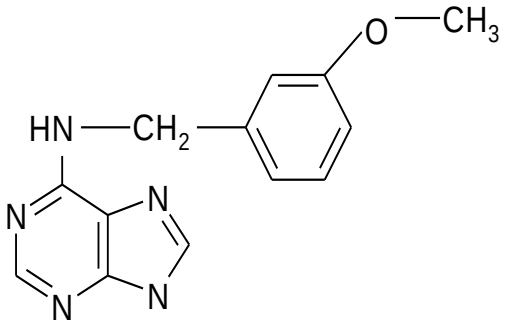
α-Naphthalene acetic acid (α-NAA)



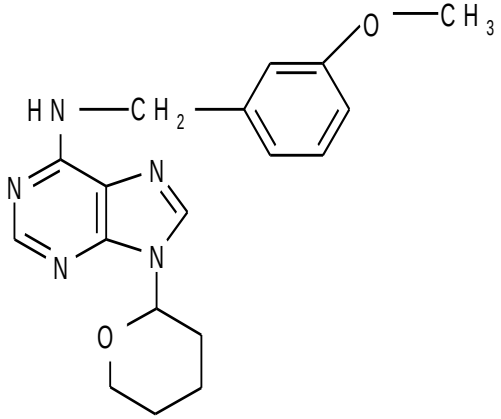
6-Benzyladenine (BA)



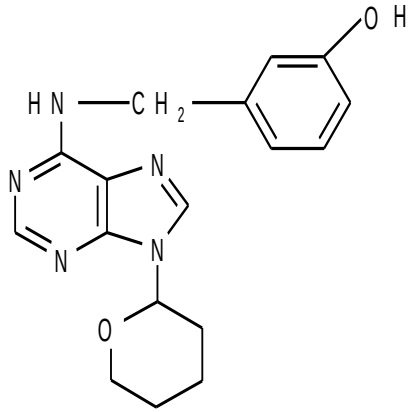
meta-Topolin (*m*T)



meta-Methoxytopolin (Me*m*T)



meta-Methoxy-9-tetrahydropyran-2-yl topolin (Me*m*TTHP)



meta-Topolin-9-tetrahydropyran-2-yl topolin (*m*TTHP)