IN VITRO REGENERATION AND SECONDARY METABOLITES IN *CYRTANTHUS* SPECIES

By

BHEKUMTHETHO NCUBE

Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy

Research Centre for Plant Growth and Development
School of Life Sciences
University of KwaZulu-Natal
Pietermaritzburg

November 2013

STUDENT DECLARATION

***In vitro* regeneration and secondary metabolites in *Cyrtanthus* species**

I, **Bhekumthetho Ncube**, student number: **209522727** declare that:

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

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DECLARATION BY SUPERVISORS

We hereby declare that we acted as Supervisors for this PhD student:

Student's Full Name: Bhekumthetho Ncube

Student Number: 209522727

Thesis Title: *In vitro* regeneration and secondary metabolites in *Cyrtanthus* species

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.

SUPERVISOR: PROFESSOR J VAN STADEN

CO-SUPERVISOR: PROFESSOR JF FINNIE

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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:

Ncube, B., Finnie, J.F., Van Staden, J., 2012. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. *South African Journal of Botany* 82, 11-20.

Contributions: Review of literature and manuscript preparation were performed by the first author under the supervision of the last two authors.

Publication 2:

Ncube, B., Finnie, J.F., Van Staden, J., 2013. Dissecting the stress metabolic alterations in *in vitro Cyrtanthus* regenerants. *Plant Physiology and Biochemistry* 65, 102-110.

Contributions: Experimental work and manuscript preparation were performed by the first author under the supervision of the last two authors.

Publication 3:

Ncube, B., Finnie, J.F., Van Staden, J., 2014. Carbon-nitrogen ratio and *in vitro* assimilate partitioning patterns in *Cyrtanthus guthrieae* L. *Plant Physiology and Biochemistry* 74, 246-254.

Contributions: Experimental work and manuscript preparation were performed by the first author under the supervision of the last two authors.

Publication 4:

Ncube, B., Finnie, J.F., Van Staden, J. *In vitro* regeneration of *Cyrtanthus* species: Ornamental plants with medicinal benefits. *Scientia Horticulturae* (Under review)

Contributions: Experimental work and manuscript preparation were performed by the first author under the supervision of the last two authors

Publication 5:

Ncube, B., Rárová, L., Nair, J.J., Finnie, J.F., Van Staden, J. Seasonal pharmacological properties and alkaloid content in *Cyrtanthus contractus* (Manuscript in preparation)

Contributions: Plant collection was done by the first author with the assistance from the third author. The second author helped with the Calcein AM assay for cytotoxicity. The rest of the experimental work and manuscript preparation were performed by the first author under the supervision of the last two authors

Signed……

BHEKUMTHETHO NCUBE

Declaration Publications FHDR 22/05/08 Approved

CONFERENCE CONTRIBUTIONS

Ncube, B., Finnie, J.F., Van Staden, J., 2012. *In vitro* propagation of *Cyrtanthus* species. 38th Annual Conference of the South African Association of Botanists (SAAB)-Plants and Society: 15-18 January 2012. University of Pretoria, Pretoria, South Africa. *South African Journal of Botany* 77: 510-580.

Ncube, B., Finnie, J.F., Van Staden, J., 2013. Stress metabolic alterations in *in vitro Cyrtanthus* regenerants. 39th Annual Conference of the South African Association of Botanists (SAAB)-Green is gold: 21-24 January 2013. Drakensberg, South Africa. *South African Journal of Botany* 86: 155-156.

ABSTRACT

Perennial geophytes form part of the diversified flora in southern Africa. The traditional medicinal system, integrates various plant components in the treatment of diverse ailments. In South Africa, bulbs are part of the extensively exploited floral resources for traditional medicine purposes. The reason for selective preference of certain groups of plant species or taxa, particularly geophytes, is rooted primarily on the beliefs that potent constituents are from the underground plant parts. The great surge of public interest in the use of plants as medicines however, assumes that plants will be available on a continuing basis. A vastly increasing human population coupled with the rapid degradation and fragmentation of natural habitats, exacerbate the threats posed by increasing demand on floral resources. The highly endemic members of the genus *Cyrtanthus*, most of which have limited geographic distribution ranges, are increasingly exploited for traditional medicines in South Africa. Bulbs of this species are the most preferred part for medicinal use, leading to the destructive harvesting of these plants. This form of plant harvesting poses threats to the long term sustainability of these plant resources in their natural habitats. Although sustainable harvesting of plant resources should be within the limits of their capacity for self-renewal, this seldom occurs owing to indiscriminate and destructive harvesting by commercial medicinal plant gatherers. As a consequence of this and other factors, intensive population decimation of a number of *Cyrtanthus* species is now evident and widespread, with some species threatened with extinction. The extinction of these species could lead to, in addition to the undesirable loss of genetic variability, loss of potential therapeutic agents. Conservation of these plant resources is therefore essential. The aim of this study was to establish efficient *in vitro* regeneration protocols for three threatened *Cyrtanthus* species (*C. contractus, C. guthrieae* and *C. obliquus*) endemic to southern Africa and explore the possible potential of improving the quality and quantity of bioactive secondary metabolites in culture.

In vitro cultured twin-scale explants of the three selected *Cyrtanthus* species using different concentrations and combinations of 6-benzyladenine (BA) (0, 1.1, 4.4, 6.7, 8.9 µM) and naphthalene acetic acid (NAA) (0, 0.5, 1.1, 2.7 µM) in a 4 x 5 factorial treatment structure, established different optimal PGR combinations for shoot

regeneration for each species. The highest shoot induction responses were obtained on MS medium with 4.4 µM BA/1.1 µM NAA for *C. contractus* and *C. guthrieae* and 6.7 µM BA/2.7 µM NAA for *C. obliquus*. The low concentration level of PGR requirements for shoot regeneration in *C. contractus* and *C. guthrieae* explants may suggest that the two species contain high enough endogenous hormones to induce shooting compared to those of *C. obliquus*. When the effect of different types and concentrations of cytokinins (CKs) [BA, kinetin (Kin), *meta*-topolins (*m*T), zeatin (ZT) and thidiazuron (TDZ)] on shoot multiplication were evaluated, 5 µM TDZ, 10 µM TDZ and 10 µM BA for *C. guthrieae*, *C. contractus* and *C. obliquus* respectively, were established as the optimum for shoot proliferation in each respective species. These results indicate, TDZ, a characteristically inexpensive CK, to be highly potent and effective in shoot proliferation of *C. guthrieae* and *C. contractus*. In terms of visual quality, shoots obtained from media supplemented with Kin and *m*T resulted in the best quality shoots in all three species at all concentrations tested. Furthermore, Kin also exhibited some auxin-like activitty by inducing rooting and callus on *C. contractus* and *C. guthrieae* cultures. The regenerated organogenic calli from *C. guthrieae* explants produced the optimum number of shoots through indirect organogenesis when transferred to MS medium supplemented with a combination of 0.1 µM picloram and 0.01 µM BA. An almost two-fold shoot proliferation frequency was obtained when the resulting callus-derived microshoots where subsequently transferred to the optimised shoot proliferation medium for the species. Regenerated shoots for all species were rooted successfully on half- and full-strength MS media without plant growth regulators, transferred to organic soil mix, and successfully acclimatised in greenhouse conditions. The developed micropropagation protocols provide a rapid and cost effective way of conservation, domestication and commercial cultivation of *Cyrtanthus* species.

The levels of proline and polyphenolic compounds measured at intervals of three, four and five weeks from initial plantlet culture under different levels of salinity and osmotic regimes, increased in a stress-dependent pattern. The levels of these metabolites also showed a significant increase with an increase in the duration of plantlets under stress conditions. The highest proline concentration (9.98 µmol g^{-1} FW) was recorded in *C. contractus* at 300 µM NaCl after five weeks. The high level of total polyphenolic compounds (147 mg GAE g^{-1} DW) for the same species was

however, recorded in the 150 µM NaCl stress treatment. The activity of proline dehydrogenase (PDH) (EC 1.5.99.8) was shown to decrease with an increase in proline levels from week three to week five in almost all stress conditions evaluated. The high levels, particularly of phenolic compounds obtained under osmotic and salinity stress conditions in this study present a promising potential for manipulating culture and/or growing conditions for improved secondary compound production and hence medicinal benefits. In a study of the growth dynamics and patterns of assimilate partitioning to primary and secondary metabolites in response to varying levels and combinations of C (carbon) and N (nitrogen) in the culture media of *Cyrtanthus guthrieae*, relative growth rate (RGR) increased proportionally with an increase in C concentrations up to 88 mM sucrose (0.58 d^{-1}) beyond which it was hardly influenced by further increases in C. In C-limited media regimes with growth saturating N conditions, alkaloid accumulation became favoured while polyphenol content increased with an increase in C levels in the medium, a characteristic pattern that appeared to be less influenced by the amount of N. Of the primary metabolites, only proteins showed small significant variations across different media treatments, with starch and soluble sugars increasing proportionately with C levels. From a medicinal perspective, with regard to polyphenolic compounds in *C. guthrieae*, growth media conditions that allow for high levels of C pools in the tissue would thus be favourable for the enhanced synthesis of this group of compounds. The medium conditions with 175 mM sucrose and 10.3 mM NH_4NO_3 gave the highest total polyphenol, flavonoid and proanthocyanidin levels with a moderate growth rate.

Pharmacological evaluation of the monthly collected *C. contractus* bulbs indicated some impressive bioactivities particularly the cytotoxicity effects against human cancer cell lines and enzyme inhibition (AChE and COX) by the extracts collected in certain months of the year. Of notable interest were the cytotoxicity effects, AChE and COX enzymes inhibitory activity of the extracts collected in May and September. Similarly, some extracts from *in vitro* precursor-fed plantlet and callus cultures demonstrated some excellent bioactivity, against COX and AChE enzymes. The results obtained from this study also reflect on the involvement of the environment in the quality of the extracts produced on a month to month basis and further suggest the importance of coinciding collection and use of plant extracts with the best time of the year or month. The good AChE and COX enzyme inhibitory activity by some of

these extracts is of significant importance in the treatment of Alzheimer's disease and neuroinflammation. The extracts represents an important component of traditional medicine.

ACKNOWLEDGEMENTS

This thesis represents a milestone in a journey that I wouldn't, in anyway, have travelled solo. The completion of this thesis is a reflection of not only my individual effort and input but rather a result of a collective effort of several people who contributed in various ways for me to accomplish this work. This journey would not have been possible without their guidance, support, patience and encouragement.

My sincere gratitude goes to my supervisor Professor J. Van Staden for the invaluable guidance, support, constructive criticism and feedback that he rendered in each step of this research work. I will always treasure the knowledge and skills he imparted to me in coming up with this piece of work and in shaping the person I am today. I would also like to thank Prof Van Staden for the financial support during the course of my study.

To my co-supervisor Professor J.F. Finnie, whose 'maddening' attention to detail drove me to finally learn to not only read on the lines; every interactive discussion we had towards this research work was incredible. The motivational approach, coupled with his incomparable critical views, and encouragement, contributed immensely to this output. I sincerely appreciate his guidance and meticulous attention to detail.

I am delighted to express my gratitude to my research committee members Dr. A.R. Ndhlala, Dr. J. Chukwujekwu and Dr. J.J. Nair for the great ideas they shared with me during my research meetings.

The cytotoxicity work presented in this thesis was made possible with the expertise and help of Dr. Lucie Rárová of the Centre of the Region Haná for Biotechnological and Agricultural Research, Palacky´ University, Olomouc, Czech Republic. I would like to express sincere thanks to her help with this assay. This research would not have been possible without financial assistance from the University of KwaZulu-Natal.

I am also delighted to express my sincere appreciation to Mrs A. Young (UKZN botanical gardens horticulturalist) and Dr. C.J. Potgieter (Bews Herbarium) for their assistance in plant identification and voucher specimen preparations.

Special mention goes to the tireless and efficient administrative team, Mrs L. Warren and Mrs J. Magnussen who kept various activities running smoothly in the Centre. The technical assistance provided by Mr Hampton and his team in the use of some equipment is greatly appreciated. I acknowledge the pleasure of working with the outstanding members of the Research Centre for Plant Growth and Development (RCPGD) and in particular the following friends and colleagues, Dr. A.R. Ndhlala, Dr. M. Moyo, Dr. R.B. Mulaudzi, Mr. A. Okem, Miss B. Madikizela, Dr. J.J. Nair, Dr. M.G. Kulkarni and Dr. P. Baskaran for their various academic and social contributions. My experience at the Centre has been nothing short of amazing.

Last but certainly not least, my deepest gratitude goes to where most of the basic source of my life energy resides, family. To Keitumetsi, Nicole and Shaun thank you for your incomparable love, support and encouragement. You guys are unique in the very many respects, and the stereotype of a ‘perfect’ family in a lot more ways. Nicole, you kept me company on the day I put the final critical touches on that paper and you were a motivation and an inspiration. To my mom, Nondulo and the rest my family members for their support and encouragement, thank you very much. This thesis is dedicated to you Shaun, for joining us during the critical stages of this journey, for your fighting zeal for life and for giving me reason to continue looking forward to the future. I love you all.

Finally, I thank God for guiding me through everything.

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2,4-D	2,4-Dichlorophenoxyacetic acid	Kin	Kinetin
AA	Arachidonic acid	LCE	Leucocyanidin equivalents
AChE	Acetylcholinesterase	LSD	Least Significant Difference
AD	Alzheimer's disease	Me*m*TR	*meta*-Methoxytopolin riboside
AIDS	Acquired Immune Deficiency Syndrome	MFC	Minimum fungicidal concentration
ANOVA	Analysis of variance	MH	Mueller-Hinton
ATCC	American Type Culture Collection	MIC	Minimum inhibitory concentration
ATCI	Acetylthiocholine iodide	MS	MURASHIGE and SKOOG (1962)
ATP	Adenosine Triphosphate	*m*T	*meta*-Topolin
BA	6-Benzyladenine	*m*TR	*meta*-Topolin riboside
BCG	Bromocresol green	NAA	α-Naphthalene acetic acid
BSA	Bovine serum albumin	ND	Not determined
CBN	Carbon/Nitrogen Balance	NIA	Nitrate reductase
CEM	T-lymphoblastic leukaemia cells	NSAIDs	Nonsteroidal anti-inflammatory drugs
CK	Cytokinin	NU	Bews Herbarium
CNS	Central nervous system	PAL	Phenylalanine ammonia-lyase
COX	Cyclooxygenase	PAL_2	Phospholipase A_2
CRD	Complete randomised design	PCM	Protein Competition Model
CTE	Catechin equivalents	PEG	Polyethylene glycol
DDI	Distilled deionised water	PGE_2	Prostaglandin E_2
DMEM	Dulbecco's modified Eagle's culture medium	PGH_2	Prostaglandin H_2
DMRT	Duncan's multiple range test	PGI_2	Prostaglandin I_2
DPM	Disintegrations per minute	PGR	Plant growth regulators
DSMO	Dimethylsulfoxide	PO	Peroxidase
DTNB	5,5-Dithiobis-2-nitrobenzoic acid	PPF	Photosynthetic photon flux
DW	Dry weight	PPFD	Photosynthetic photon flux density
dZ	Dihydrozeatin	PVPP	Polyvinylpolypyrrolidone

FBS	Foetal bovine serum
FW	Fresh weight
GAE	Gallic Acid Equivalents
GE	Glucose equivalent
GST	Glutathione -S-transferase
HIV	Human immunodeficiency virus
IAA	Indole-3-acetic acid
IAOx	Indole-3-acetaldoxine
IBA	Indole-3-butyric acid
INT	*p*-Iodonitrotetrazolium chloride
iP	*N*6-Isopentenyladenine
IPA	Indole-3-pyruvic acid
RCPGD	Research Centre for Plant Growth and Development
ROS	Reactive oxygen species
SAAB	South African Association of Botanists
SOD	Superoxide dismutase
TAM	Tryptamine
TCA	Trichloroacetic acid
UKZN.	University of KwaZulu-Natal
UV	Ultraviolet
WHO	World Health Organisation
YM	Yeast malt
ZT	Zeatin

CHAPTER 1
Introduction and Literature Review

1.1. Introduction

The complex, integrated and mutualistic interaction of structural and functional components and processes of different ecological systems on earth allows for persistence of life and nature. Although ecosystems are defined by the network of interactions among organisms, and between organisms and their environment (**CHAPIN et al., 2002**) and are usually fragmentised into ecologically homogeneous units of any size, the entire planet is also regarded as an ecosystem. In this context, **PRIMACK and CORLETT** (**2005**) describe plants as fundamental structural and nutrient-sequestering components of most stable ecosystems. Through the process of photosynthesis, plants harness solar radiation energy to reduce inorganic carbon sources into chemical energy forms (carbohydrates) that are utilised and circulated within ecosystems. Plants, as part of individual species that constitute terrestrial biodiversity, have received growing appreciation of their role in the functioning of ecosystems and how these functions provide services that are vital to human welfare (**HAMILTON and HAMILTON, 2006**). As a resource, plants provide the predominant ingredients of the various facets of human life. Not only do plants provide food and materials essential for human existence (**KIER et al., 2005**), they also contribute ingredients for human medicines. For this reason, the scale and intensity of human interactions with plants continue to be of such magnitude that wide-scale habitat loss, degradation and fragmentation, with subsequent loss of plant species and genetic variability progressively increases with time (**HEYWOOD and IRIONDO, 2003**). Human activities are highly variable in their influence on plant biodiversity, leaving no part of the world that can be considered as truly 'undisturbed' (**HEYWOOD and IRIONDO, 2003; GIAM et al., 2010**). Numerous studies describe logged forests as being able to retain much of their original biodiversity compared to human impact on forests despite the severe damage that logging can inflict on the forest ecosystem (**WHITMORE and SAYER, 1992**, **MEIJAARD et al., 2005**). The ever increasing human population exerts heavy demand on land for urbanisation and agriculture and accelerates changes in climate with subsequent broad-scale global

land-cover transformation that increases plant species extinction rates. In spite of the frightening ramifications for humanity as a result of the loss of plant biodiversity and the advances in plant sciences made to date, a number of plant resources continue to be exploited in their natural habitats in the wild. In the absence of, or if potential conservation measures are not matched with resource utilisation/depletion, a number of plants would be at risk of over-exploitation and extinction. Their conservation thus needs renewed attention. In South Africa, perennial geophytes of the genus *Cyrtanthus* form part of the rich floral diversity and are extensively exploited for both ornamental and medicinal purposes unmatched with the efforts to cultivate and domesticate them.

1.2. Morphology, distribution and utilisation

1.2.1. The Amaryllidaceae Family

The Amaryllidaceae family is a group of herbaceous, perennial, bulbous and/or, less commonly rhizomatous flowering plants included in the monocotyledonous order Asparagales. It consists of about 60 genera, with over 860 species, distributed primarily in the tropical and subtropical areas of the world. Earlier studies suggested the centre of diversity of Amaryllidaceae to be in the Southern Hemisphere, especially South Africa and South America, with some genera also found in the Mediterranean and temperate regions of Asia (**MEEROW and SNIJMAN, 1998**). Molecular evidence indicate an African origin for the family, with later dispersal to other continents, suggesting that South America maybe a centre of secondary diversification (**ITO et al., 1999**). Based on nrDNA ITS sequences, **MEEROW and SNIJMAN** (**2001**) inferred from their findings that a great deal of the diversity of the family in the Americas is recent, and that the American Amaryllidaceae may have been reduced to peripheral isolates sometime after its initial entry and spread through the Americas. Members of this family possess extraordinary diversity in reproductive traits, even among closely related species. This variation is a result of the evolutionary lability of reproductive characters, implying the existence of diverse functional abilities for achieving mating and fertility (**SINGH, 1972; LLOYD et al., 1990**). Tristyly, a rarer form of heteromorphic incompatibility in plants, is one of the sexual polymorphic characteristic that have been identified in some populations of

the genus *Narcissus* (**BARRETT et al., 1997**). About 85% of the *Narcissus triandrus* populations contain three discrete floral morphs that differ from one another in the sequence in which the stigmas and anthers are presented within flowers (**BARRETT et al., 1997**). The floral diversification has resulted in contrasting suites of floral characters associated with different pollinator groups.

Over the years and to date, members of this family have been exploited for human benefit in a variety of ways. World-wide, members of the Amaryllidaceae have their greatest economic value as ornamentals. In addition, a huge number of these plant species are used and traded in traditional medical practices (**DU PLESSIS and DUNCAN, 1989; MANDER, 1998**). Amaryllidaceae species are a valuable source of alkaloids (**VILADOMAT et al., 1997**), many of which are used commercially in the modern medicinal system.

1.2.2. The genus *Cyrtanthus*

The genus is endemic to the southern and eastern parts of Africa and has distribution across all provinces in South Africa, with the highest concentration of species in the southern parts of the Eastern Cape. There are about 60 species in the genus *Cyrtanthus*, more than 90% of which are concentrated in South Africa (**REID and DYER, 1984; DUNCAN, 2007**). Its diversity decreases towards the northern parts of the country but its distribution extends into Zimbabwe and further north into east Africa. *Cyrtanthus* species are perennial bulbs. Within their ecologically diverse niches, the species may be evergreen, growing either in winter or summer. According to phylogenetic and biogeographic relationship studies, the genus comprises three poorly to well-supported major lineages: a predominantly Afrotemperate lineage, largely restricted to seasonally moist sites in summer rainfall areas of southern Africa, a subtropical lineage found mostly in non-seasonal rainfall regions, often in dry habitats, and a Cape Floristic Region centred lineage in which most species are concentrated in the summer-dry to non-seasonal rainfall southwest of the Cape (**SNIJMAN and MEEROW, 2010**). The leaves differ significantly among species, from slender to strap-shaped. It is the most diverse in terms of floral colour, morphology and orientation (**Figure 1.1)** of any genus in the Amaryllidaceae family

and this has seen numerous species attracting great ornamental value in the floriculture industry (**DU PLESSIS and DUNCAN, 1989**). A few species have actinomorphic flowers, but the majority deviate from this arrangement by the curvature of the perianth tube, a feature usually associated with their nodding habit (**DU PLESSIS and DUNCAN, 1989**). The bulb is tunicate and usually produces one hollow (in some species solid) peduncle. Most species have leaves during flowering. The perianth is usually tubular for more than half of its length and the ovary contains numerous ovules (**ISING, 1991**).

Most of the species do well as garden and pot plant flowers. Because of its floral diversity, the genus exploits a variety of pollinators. The species produce flattened, winged phytomelanous seeds. Several species in the genus are also highly sought after in traditional medicine in South Africa (**NORDAL, 1979; REID and DYER, 1984; DUNCAN, 1990a; HUTCHINGS et al., 1996**). Many species in the genus are known for their extremely rapid flowering response to fire, hence the common name, "Fire Lily" for several species. Some of the species, such as, *C. ventricosus* and *C. odorus* are known to flower only after fires (**REID and DYER, 1984; KEELEY, 1993**). **BRYAN** (**1989**) describes this fascinating, but ephemeral behaviour as the one dictating the slow progress of studying *Cyrtanthus* in the wild. Conversely to its floral morphological diversity, the genus shows great consistency in chromosome number, with 2*n*=16, characteristic of most, if not all, of the species (**ISING, 1970; STRYDOM et al., 2007**). Despite this consistency, however, numerical unbalance in combination with hybridity have been noted in several species causing a high frequency of structural chromosome changes (**ISING, 1969**) that may result in altered karyotypes (**ISING, 1967**). On investigating the karyotypes of 17 *Cyrtanthus* species, **WILSENACH** (**1963**) characterised the species with long, narrow perianth tubes as having asymmetrical karyotypes, and those with the broadly funnel-shaped tubes to have the more symmetrical karyotypes. Although this is generally true, a few exceptions to this rule, however, have been noted, for example, *C. herrei, C. flanagani* and *C. contractus* have narrow perianth tubes despite having the more symmetrical karyotypes, whereas *C. breviflorus* and *C. erubescens* have small funnel-shaped flowers while exhibiting asymmetrical karyotypes.

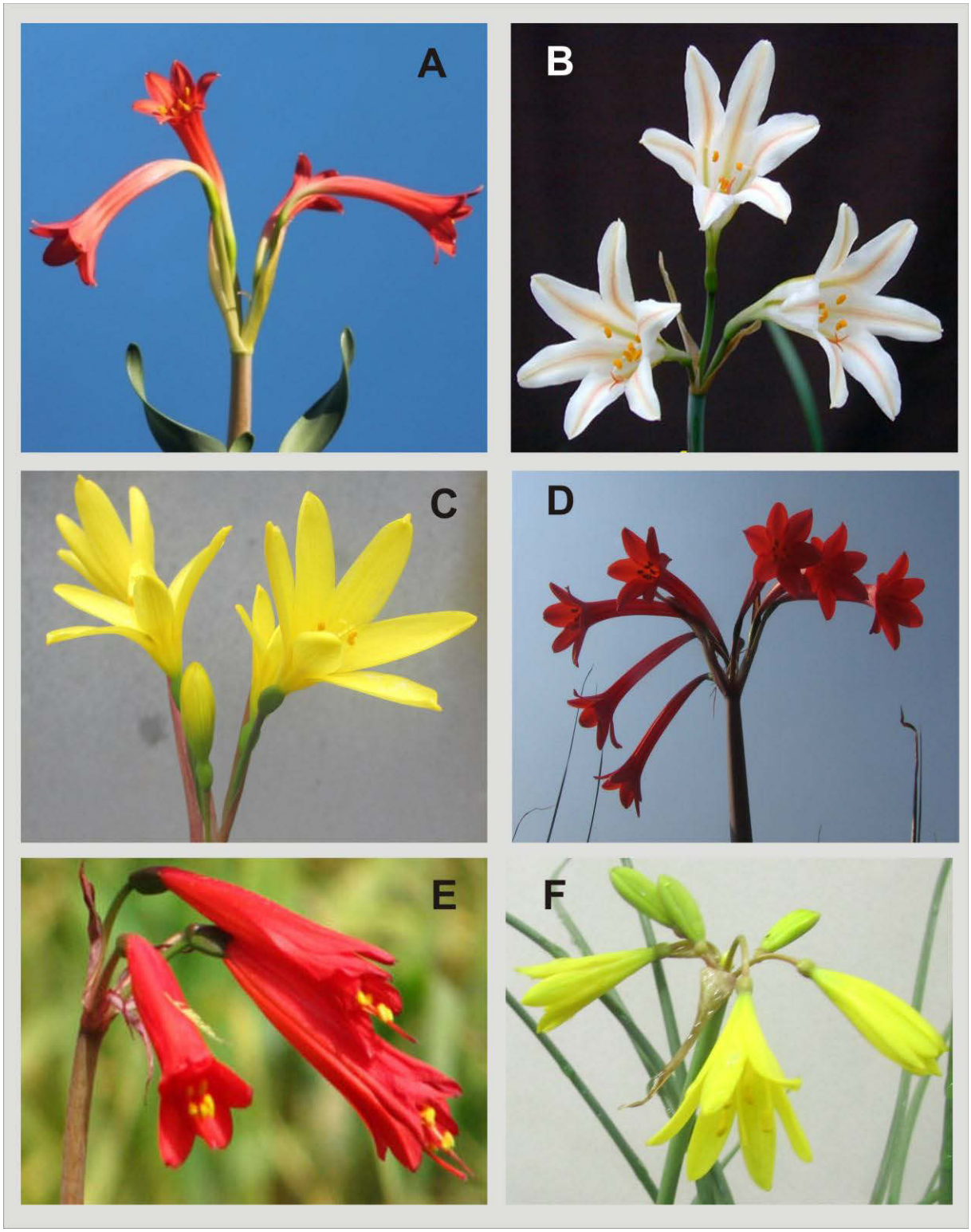


Figure 1.1. Representatives of *Cyrtanthus* species showing various floral colours and morphological diversity (**A**) *C. spiralis* (**B**) *C. loddigesianus* (**C**) *C. mutans* (**D**) *C. contractus* (**E**) *C. brachyscyphus* (**F**) *C. breviflorus*

Virtually every member of the genus exhibits some degree of horticultural potential. However, due to their rather delicate nature, and the difficulty in cultivating many of the species, their greatest potential lies in their use as container subjects (**DUNCAN, 1990a**). Furthermore, enormous potential lies in the field of hybridisation, as subjects for both container and garden cultivation, as well as for the cutflower market.

1.2.2.1 *Cyrtanthus contractus* N.E. Br.

Cyrtanthus contractus is one of the most widely distributed species in South Africa. It is found in higher concentrations in the Eastern Cape where it grows in the inland areas (**BRYAN, 1989**). The plant grows to a height of about 40 cm and usually flowers in spring and during the early summer. The species has pink to somewhat red coloured flowers. It occurs in various habitats, sometimes in open grassland where it flowers in October or earlier if stimulated by fire (**DU PLESSIS and DUNCAN, 1989**). Bulb infusions of *C. contractus* are used in traditional Zulu medicine as sprinkling protective charms against storms and evil (**HUTCHINGS et al., 1996**)

1.2.2.2. *Cyrtanthus guthrieae* L. Bolus.

Cyrtanthus guthrieae is a deciduous, summer or winter-growing geophyte with an average height of 15-25 cm. **DUNCAN** (**2003**) describes *C. guthrieae* as the rarest, and certainly one of the most beautiful, species in the genus. The species has an extremely localised known distribution close to the southern Cape Atlantic coast, and grows on sharply drained, nutrient deficient, acid sandstone mountain slopes. Although it's natural growing habitat is mainly in the winter rainfall, its active growth period under cultivation is highly erratic, producing new leaves either in spring or autumn, or occasionally remaining evergreen for up to a year, and then becoming dormant for an extended period. The species is highly variable with regard to overall flower shape, as well as in the colour and shape of the perianth segments, from bright scarlet with broadly lanceolate segments to reddish-orange flowers and

broadly obovate segments (**SNIJMAN and VAN JAARSVELD, 1995; DUNCAN, 2003**). Flowers of this species are pollinated by the large-winged mountain pride butterfly, *Aeropetes tulbaghia* L., whose abundant numbers in autumn coincide with its flowering period in the wild (**JOHNSON and BOND, 1994; JOHNSON, 2010**). Although *C. guthrieae* flowers profusely following fire, the species appears not to be dependent on fire for flowering to occur (**DUNCAN, 2003**). The bulbs of this species always seem to be solitary and reproduce less often by offset formation.

1.2.2.3. *Cyrtanthus obliquus* (L.f.) Ait.

A robust evergreen geophyte that grows to a height of up to 50 cm. The species seem to be specialized for arid conditions and usually grows in dry rocky areas. The large bulbs and thick leaves are adapted to withstand drought for long periods of time (**ISING, 1970; VOGEL and MÜLLER-DOBLIES, 2011**). Their generation time is very long and may require 5 to 10 years from seed to flowering even under favourable natural conditions. The leaves are blunt tipped and twisted, with yellow-red edged margins. The species produces yellowish green and red flowers, with a waxy bloom and tipped green lobes in an inflorescence hanging from hollow stems. The twisted evergreen glamorous foliage combines magnificently with the sturdy peduncle of pendulous yellow, red and green flowers. Its flowering season extends from August through to February (**REID and DYER, 1984**; **POOLEY, 1998, DUNCAN, 1990b**). In South Africa, *C. obliquus* is distributed in the Western Cape, Eastern Cape and KwaZulu-Natal provinces.

1.2.3. Horticultural value and potential of *Cyrtanthus* species

The richness and uniqueness of the southern African flora has attracted local and international interest from the horticultural industry. In their list of indigenous southern African plant species of commercial interest in the florist trade, **BROWN and DUNCAN, 2006; REINTEN et al.** (**2011**) highlight *Cyrtanthus* among other members of the Amaryllidaceae family as being of historic and current commercial interest. Under this listing, the authors categorise *Cyrtanthus* species' use and/or potential use to be high as fresh and potted flowers. Their primary horticultural uses

are as container plants and in gardens with warm (non-freezing) growing conditions (**THERON and DE HERTOGH, 2001**). Based on visual appearances only, *Cyrtanthus* species have been cited as one of the most likely candidates with potential as potted plants commercially on the international market (**REINTEN et al., 2011**). The variety of colours and diversity of floral forms make the species an obvious choice for cut flower production. **DALY and HENRY** (**2009**) reported that potted geophytes as winter-blooming house plants are in demand in the USA market and cited *Cyrtanthus* as among these. Commercially, *Cyrtanthus* is grown in South Africa, New Zealand, United Kingdom and in the Netherlands (**CLARK et al., 2000; DUNCAN, 2010**). *Cyrtanthus* flowers, originally from South and East Africa, have been grown for many years in New Zealand as cut flowers, with new hybrids produced each year to widen the choice for the consumer. They are planted as small, non-flowering bulbs and are either grown in the field or under protected cultivation for 2-3 years before they are replanted for flowering or sold as flowering-grade bulbs (**VAN EPENHUIJSEN, 2005**). The red or scarlet type of *C. elatus* is mainly grown in New Zealand for the domestic and export markets.

The rapidly growing interest in *Cyrtanthus* as a cut flower is pioneered by New Zealand. A number of successful research efforts focussing on the vase life, storage and postharvest handling of *Cyrtanthus* as cut flowers have been reported (**CLARK et al., 2000; EASON et al., 2002; DEBENHAM et al., 2009**). In two experiments the vase life of the orange flowered cultivar was 15 and 18 days in water, whereas in other experiments the maximum vase life of the red flowered cultivar in water was 11 days. Treatment of less mature stems of the orange flowered cultivar with a double dose of Chrysal-SVB increases the vase life by 4 days (**EASON et al., 2002**), a significant contribution to marketability of the flowers. The species appear to be tolerant to a wide range of growing temperatures, with 18 °C and above considered somewhat optimal although flower quality can be affected at extreme temperatures (**CLARK et al., 2000**). Variations in the flowering periods of different *Cyrtanthus* species allows for the availability and supply of beautiful flowers all year round. Most species flower profusely following fire. Some species, especially *C. brachyscyphus*, *C. mackenii*, *C. macowanii* and *C. staadensis* flower regularly and during specific times of the year. *Cyrtanthus elatus* is an evergreen species that flower sporadically throughout the year, but more in the early summer while *C. contractus* and *C. tuckii*

flower in their natural habitats for a short period in early spring (**NIEDERWIESER et al., 2000**)

1.2.3.1. Hybridisation potential

In order to meet the rapidly changing insatiable market demands of the floriculture industry, increasing premium on flower novelty becomes inevitable. Variation in floral attributes within commercially cultivated or potential candidate species can be achieved through intra and intergeneric hybridisation of species, with significant success scored through careful consideration of market demands. A number of *Cyrtanthus* species are pleasantly fragrant, a characteristics which would greatly enhance the appeal of hybrid cut flowers if combined with the requirements such as flower longevity, colour and stem length.

Owing to the difficulties in manipulating evergreen plants, **DUNCAN** (**1990a**) and **NIEDERWIESER et al.** (**2000**) advocated that breeding *Cyrtanthus* should be aimed at hybrids with seasonal flower initiation. A number of successful hybrids have been developed over the years and possible improvement efforts continue to date. Because of the large number of extremely diverse floral species and the ease with which hybrids can be produced (**ISING, 1970**), *Cyrtanthus* constitutes valuable material for the production of new ornamental varieties. Breeding for regular flowering had been possible for a number of species, for example, *C. flanaganii* cultivated at Roodeplaat had problems flowering. However, the hybrids *C. mackenii* x *C. flanaganii* and *C. macowanii* x *C. flanaganii* flowered successfully. Furthermore, preliminary observations indicated that some species could be associated with regular flowering when used in crosses e.g., *C. brachyscyphus*, *C. galpinii*, *C. eucallus* and, *C. macowanii* (**NIEDERWIESER et al., 2000**). In addition the smaller species, such as *C. parviflorus*, *C. mackenii*, *C. breviflorus* and *C. eucallus* and the hybrids between them flower after 2-3 years and are easier to cultivate (**ISING, 1970**). Some of the notable improvements from *Cyrtanthus* hybrids are: relatively small bulbs; more than 50% of the crosses resulting in hybrids with an acceptable rate of propagation; flower colour of most hybrids are brighter and mostly red; hybrids of *C. flanaganii* crosses, had a pleasant fragrance and an improved vase life

of flowers (**COERTZE and LOUW, 1990; DUNCAN, 1990b; NIEDERWIESER et al., 2000; EASON et al., 2002; DEBENHAM et al., 2009**). Of the species most often used as pollen parent, *C. flanaganii*, *C. galpinii*, *C. loddigesianus*, *C. montanus*, *C. obliquus*, and *C. sanguineus* appeared to give desirable characteristics (**NIEDERWIESER et al., 2000**). One of the striking examples to note is a resulting tetraploid (4n) hybrid cross between *C. parviflorus* (2n) and *C. mackenii* var. *cooperi* (2n) which gave rise to a large variation in red colour forms/shades. When F_1 individuals were self-pollinated, flowers segregated in colour ranging from deep yellow to pure white (**ISING, 1966**). The large variation in flower colour entails the existence of different combinations of the alleles for red and yellow, an aspect of which makes *Cyrtanthus* hybridisation holds prospects in the floriculture industry. It is evident, therefore, that an almost limitless potential exists for developing new *Cyrtanthus* hybrids for the trade in potted, garden and cut flower markets. For the attractive species, currently not on the market because of challenges relating to propagation, cultivation, irregular flowering, short flowering periods, hybridisation remains a potential solution for their improvement. **Table 1.1** shows some of the successful *Cyrtanthus* hybrids on the market, including cultivated species.

Table 1.1. Commercially cultivated *Cyrtanthus* flowers including those with commercial potential and hybrids

Species/Hybrid	Flower characteristics	Flowering period	Flower subject	Reference
C. brachyscyphus Baker	An evergreen species with bright flowers that vary in shades of red and orange. Forms thick clumps	Sporadic throughout the year but mainly in summer and spring	Container and garden	**COERTZE and LOUW, 1990; DUNCAN, 1990b**
C. breviflorus Harv.	Dormant in winter but occasionally remains evergreen and gives rise to bright yellow flowers	Sporadic throughout the year but mainly in summer and spring	Container and garden	**COERTZE and LOUW, 1990; DUNCAN, 1990b**
C. clavatus (L'Hérit) R.A. Dyer	Dwarf with cream or pale pink flowers, dormant in winter	Summer	Container	**COERTZE and LOUW, 1990; DUNCAN, 1990b**
C. contractus N.E. Br.	Tall stems with large, bright red flowers	Spring and summer	Container and garden	**NIEDERWIESER et al., 2000**
C. epiphyticus J.M.Wood	Dormant in winter with pendulous reddish-orange flowers. Bulbs grow with the neck fully exposed.	Mid-summer	Container	**DUNCAN, 1990b**
C. elatus (Jacq.) Traub	Evergreen species, variable in flower form and size but very floriferous with mostly scarlet, pink and red flower types preferred for cultivation	Summer	Container, garden and cut flower	**DUNCAN, 1990b; CLARK et al., 2000; NIEDERWIESER et al., 2000; EASON et al., 2002; VAN EPENHUIJSEN, 2005; LEE et al., 2012**
C. erubescence	Tall stems with bright pink flowers	Summer	Container and cut	**NIEDERWIESER**

Species/Hybrid	Flower characteristics	Flowering period	Flower subject	Reference
Killick			flower	**et al., 2000**
C. eucallus R.A. Dyer	Evergreen and multiplies rapidly producing floriferous orange flowers. Bulbs grows with the neck fully exposed.	Summer	Container	**DUNCAN, 1990b**
C. falcatus R.A. Dyer	A remarkably tough species that goes dormant in winter	Late spring and early summer	Container and garden	**COERTZE and LOUW, 1990; DUNCAN, 1990b**
C. fergusoniae L.Bolus	An extremely attractive deciduous or evergreen species with bright red tubular flowers	Summer	Container	**DUNCAN, 1990b**
C. galpinii Baker	Dwarf, floriferous with pink or orange flowers	August and September	Container and garden	**DUNCAN, 1990b; NIEDERWIESER et al., 2000**
C. guthrieae L. Bolus	An extremely rare and beautiful species with bright red flowers that reflect a golden sheen in sunlight. Usually dormant in summer	March and April	Container	**DUNCAN, 1990b; SNIJMAN and VAN JAARSVELD, 1995; DUNCAN, 2003**
C. herrei (Leighton) R. A. Dyer	The main attraction is the evergreen, glaucous foliage. Has huge bulbs and the umbels of greenish-orange, pendulous flowers shows erratically	Summer	Container	**REID and DYER, 1984; DUNCAN, 1990b; REID, 1994**
C. loddigesianus (Herb.) R.A.Dyer	Floriferous cream-flowered species dormant in winter	Mid-summer	Container	**DUNCAN, 1990b**
C. mackenii Hook.f	Delightfully scented with numerous colour forms, dormant in winter	Mid-summer	Garden and cut flower	**DUNCAN, 1990b; CLARK et al., 2000**
C. montanus R.A.Dyer	Produces orange umbels and is usually evergreen	February and March	Container and cut flower	**DUNCAN, 1990b;**
C. obliquus (L.f.) Ait.	The twisted evergreen glamorous foliage	Spring and summer	Container and cut	**REID and DYER,**

Species/Hybrid	Flower characteristics	Flowering period	Flower subject	Reference
	combines magnificently with the sturdy peduncle or stock of pendulous yellow, red and green flowers		flower	**1984; DUNCAN, 1990b; POOLEY, 1998,**
C. obrienii Baker	Fairly attractive evergreen species with red pendulous flowers	Sporadic throughout the year but mainly in spring	Container and garden	**DUNCAN, 1990b;**
C. sanguineus (Lindl.) Walp	Floriferous evergreen produces flowers that vary in colour from bright pink to orange-red and deep red and often produces two stems. The rather distinctive leaves are dark green with purplish margins and midribs	Mid-summer	Container	**DU PLESSIS and DUNCAN, 1989; DUNCAN, 1990b; LEE et al., 2012**
C. smithiae Watt ex Harv.	Deciduous with very attractive corkscrew foliage that produces short-lived striped, white or pale pink flowers. Bulbs grow with the necks just below the soil surface	Summer	Container	**DUNCAN, 1990b**
C. spiralis Burch. ex KerGawl	Very rare in the wild and evergreen species with nicely coloured red flowers	Mid-summer	Container	**DUNCAN, 1990b**
C. staadensis Baker	Rare floriferous species with bright, orange-red flowers and dilated perianth tubes carried on long peduncles or stalks	January-March	Container and cut flower	**DUNCAN, 1990b; DUNCAN, 2007**
C. sanguineus Schonland x *C. elatus*	Evergreen and multiplies rapidly with mature bulbs often producing two inflorescences each with up five bright red colours	Summer	Container, garden and cut flower	**COERTZE and LOUW, 1990; DUNCAN, 1990c; LEE et al., 2012**
C. elatus x *C. fergusoniae*	Evergreen, highly floriferous hybrid with long-lasting bright red tubular flowers. It has rapid vegetative reproduction characteristic	Spring and early summer	Container and cut flower	**DUNCAN, 1990c**
C. elatus x *C. montanus*	Evergreen, long-lasting floriferous with inflorescence colour of *C. elatus* but the	Mid-summer	Container and cut flower	**COERTZE and LOUW, 1990;**

Species/Hybrid	Flower characteristics	Flowering period	Flower subject	Reference
	shape of *C. montanus*.			**DUNCAN, 1990c**
C. montanus x *C. guthrieae*	Dwarf, evergreen and rapidly reproducing hybrid that combines the striking colour of *C. guthrieae* and the flower shape of *C. montanus*	Mid-summer	Container	**COERTZE and LOUW, 1990; DUNCAN, 1990c**
C. smithiae x *C. elatus*	Summer growing hybrid that exhibit the corkscrew foliage of *C. smithiae* with beautiful pink and white-striped flowers	Early summer	Container and cut flower	**COERTZE and LOUW, 1990; DUNCAN, 1990c**
C. spiralis x C*. sanguineus*	Dwarf evergreen with dark-red, long-lasting semi-pendulous flowers. Each bulb produces two stems	Summer	Container	**COERTZE and LOUW, 1990; DUNCAN, 1990c**
*C. '*Orange Gem' × *C. eucallus*				**LEE et al., 2012**

1.2.4. Medicinal uses

The various facets of traditional lifestyles of indigenous people involves the use of biodiversity to sustain cultural identity. This has, over generations of living, created a relationship between biodiversity and human diversity, which the Declaration of Belem (1988) describes as an ‘‘inextricable link’’ between biological and cultural diversity (**POSEY, 1988**). The use of traditional medicine to maintain good health is one of the major cultural practices in which human beings have interacted extensively with biodiversity. In an African context, traditional medicine takes the form of a somewhat holistic approach that integrates spiritual, plant and animal components. Although animals and plants are integral components of this holistic approach in the treatment of many ailments (**CUNNINGHAM, 1993; BODEKER, 2004**), plants are regarded as the root of this medical practice (**GURIB-FAKIM, 2006**). The treatment or prescription may at times call for animal sacrifice but will almost always call for the use of herbal remedies (**DU TOIT, 1980**). Because of the varied and complex perceptions on the causes of ill health and social imbalances, the treatment of various human ailments follows rather diverse forms that sometimes vary between cultures and regions. The philosophy is however the same in most African communities.

Traditional medicine continues to be of major importance in the provision of primary health care in most African countries (**CUNNINGHAM, 1993**) and **WHO** (**2008**) estimates this to be 70% of the total African population. This has resulted in the loss of biodiversity. In South Africa, the genus *Cyrtanthus* forms part of the geophytes exploited from the wild for various medicinal purposes. **Table 1.2** presents the medicinal uses of *Cyrtanthus* species used in this study. Being members of the Amaryllidaceae family, the genus is a potential source of medicinal alkaloids. Due to their extensive use in traditional medicine, the populations of these species are decreasing rapidly in the wild. This loss of biodiversity not only leads to diminishing biological resources, but also a shift in cultural practices (**WIERSUM et al., 2006**). The potential loss of cultural value which has a strong link to medicinal plant biodiversity, therefore, supports the initiative for conservation of medicinal plant biodiversity in Africa (**ANYINAM, 1995; WIERSUM et al., 2006**).

Table 1.2. Ethnobotanical uses of the three *Cyrtanthus* species used in this study

Plant species	Voucher specimen	Medicinal uses
C. contractus N.E. Br.	**NCUBE 05 NU**	Infusions of the bulb are used as sprinkling charms against storms and evil (**HUTCHINGS et al., 1996**)
C. guthrieae L. Bolus	**NCUBE 06 NU**	Infusions of the bulb are used as sprinkling charms against storms and evil (**HUTCHINGS et al., 1996**)
C. obliquus (L.f.) Ait.	**NCUBE 07 NU**	Root infusions are used for stomach ache, while the crushed roots are applied over the affected area in the treatment of leprosy. Decoctions made from roots of *Capparis brassii* and part of the bulb of *C. obliquus* are taken against chronic coughs and sometimes, with the addition of warm water, as an emetic. Leaves and thin bulbs are used as an absorbent to dress wounds (**BROWNLEE, 1931; NWUDE and EBONG, 1980; BHAT and JACOBS,1995; HUTCHINGS et al., 1996**)

NU = Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg

1.2.4.1. Alkaloids and *Cyrtanthus* species

The use of alkaloids in medicinal systems from alkaloid-containing plants dates back to the beginning of civilisation (**ELIOVSON, 1967; KUTCHAN, 1995**). Being a structurally diverse group of chemical compounds, alkaloids mostly contain more than one functional group and therefore, often exhibit multiple functionalities and bioactivities. Amaryllidaceae species produce alkaloids of the norbelladine type and are an exclusive source of this type of alkaloids (**KUTCHAN, 1995**). Due to the limited taxonomic distribution of alkaloids isolated from these plant species, they are often classically grouped as Amaryllidaceae alkaloids. Since the identification of the first alkaloid, morphine, from the opium poppy (*Papaver somniferum*) in 1806 (**SOUTHON and BUCKLNGHAM, 1989**), there has been tremendous interest in alkaloids in medical sciences. The recently demonstrated potent anticancer activity of certain lycorane-type alkaloids and the selective, long acting, reversible and competitive acetylcholinesterase (AChE) inhibitory activity by galanthamine (**SRAMEK et al., 2000; HOUGHTON et al., 2006; MARCO-CONTELLES et al., 2006; MCNULTY et al., 2010**) has stimulated the clinical advances of Amaryllidaceae alkaloids and their synthetic derivatives towards the development of anti-tumour and neurodegenerative therapeutics. Galanthamine, under the generic name reminyl, became the first Amaryllidaceae alkaloid to be approved as a prescription drug in the treatment of Alzheimer's disease (**MCNULTY et al., 2007**). Although alkaloids are produced as adaptive compounds in plants, they are also thought to be involved in the biosynthesis of compounds needed for cell to cell communication within the plant (**GHOSAL et al., 1990**).

The medicinal and toxicological properties of alkaloids clearly make them important to humans. Pharmacological screening of a wide range of plant-isolated alkaloids demonstrates a diverse range of biological activities that are both beneficial and potentially detrimental to human health. Among the most beneficiary effects are: the antimicrobial, anticholinergic, antitumour, antimalarial, anti-inflammatory, antiviral, analgesic and nervous system effects (**GHOSAL et al., 1985; VILADOMAT et al., 1996; VILADOMAT et al., 1997; PHAM et al., 1998**). Phytochemical investigations within the *Cyrtanthus* genus led to the isolation of Amaryllidaceae alkaloids from *C. elatus* (**HERRERA et al., 2001**), *C. obliquus* (**BRINE et al., 2002**) and *C. falcatus*

(**ELGORASHI and VAN STADEN, 2004**) *C. contractus* (**NAIR et al., 2011**). The compounds interact effectively with AChE and are distributed within *Cyrtanthus* species. In addition, these alkaloids have demonstrated selective cytotoxicity towards certain tumour cells and hence provide an encouraging incentive for the development of potentially tumour cell-line targeted chemotherapeutics (**MCNULTY et al., 2007; MCNULTY et al., 2009**). Their distribution within *Cyrtanthus* species makes the genus a lucrative potential for the discovery and development of novel chemotherapeutics. However, the side effects, with symptoms such as, headaches, excessive salivation, nausea, dizziness, heartbeat irregularities, visual disturbances, dermatitis, and hepatotoxicity are among the severe limitations that some classes of alkaloids have in their therapeutic success (**BORES et al., 1996; HUTCHINGS et al., 1996**). The side effects are due to non-selectivity of some of these compounds. The fact that many alkaloids could be toxic provides insight into their potential function in the chemical defence arsenal of the plant.

1.3. Conservation drive

Plants and their habitats are challenged by a number of multidimensional pressures, from industrial development, environmental pollution, farming, population growth and urban expansion (**NEWTON and BODASING, 1994; FENNELL, 2002**), forces of which lead to loss of habitats and an eroded plant genetic variability. With the increased realisation that some wild species are being over-exploited, the International Union for Conservation of Nature (IUCN) set up assessment procedures for listing and categorising the status of threatened plant populations in the wild. Under this listing, **SARASAN et al.** (**2006**) reported that a total of 8 321 plant species were threatened during the period 1996-2004, with the number of those recorded as “critically endangered” increasing by over 60% during the same period. Critically endangered plants are at a high risk of becoming extinct. If a plant species becomes extinct, it disappears with its genotype, leading to a loss of genetic variability in a plant population. Loss of genetic variability is an extremely undesirable phenomenon in any ecosystem. The loss of floral biodiversity, not only threatens the survival of ecosystems, but also to a greater extent, the livelihoods of people that depend on them (**NCUBE et al., 2012**). It is for this reason that certain groups of

plants, by virtue of their economic and cultural significance, are at the risk of over-exploitation and extinction, and there needs to be renewed attention with respect to their conservation. In traditional medicine, it has been established that certain plant families, genera and species are demanded in higher proportions than others (**LAMBERT et al., 1997**). In addition, these plant families are not distributed uniformly across any given geographical locality. As a consequence, not only do some floras have higher proportions of valuable plants in demand than others, but also, certain plant families have a higher proportion of threatened species than others.

Sustainability of harvesting of medicinal plants is influenced by many factors, from both social and ecological perspectives. Many interlinked dimensions (ecological, biological, socio-cultural and economic) must all be considered in order to achieve sustainable use of medicinal plants. Medicinal bulbs are particularly in danger of loss through destructive harvesting in the wild. In some plant ecological studies, strong positive correlations were established between the plant parts harvested, harvesting methods and their impact on plant demography (**OLMSTED and ALVAREZ-BUYLLA, 1995; TICKTIN, 2004**). **JÄGER and VAN STADEN** (**2000**) attributed the increased use of the non-renewable plant materials such as roots, bulbs and bark as a major contributing factor to the decline of most species in the wild. Harvesting rates of between 5% and 15% resulted in a significant population decline in *Allium tricoccum*, a species whose underground bulbs are utilised in traditional medicine (**NAULT and GAGNON, 1993**). **ROCK et al**. (**2004**) found this species to have limited population recovery over time. Eco-geographic factors, in combination with harvesting and utilisation pressures, plays a significantly role in the susceptibility of plant population decline and extinction. Inherent characteristics such as slow growth and reproductive rates, long generation times and the production of relatively few propagules render the species especially vulnerable and adversely affected by whatever destructive harvesting method is employed by commercial gatherers (**WILLIAMS et al., 2013**). Habitat fragmentation on the viability of species populations with endemic and restricted distributions varies according to the degree of fragmentation, and the species characteristics, with regard to seed dispersal, mating system, and life forms (**YOUNG et al., 1993; WILLIAMS et al., 2013**). The risk of local extinction of such species generally becomes higher due to the loss of

genetic diversity and are more likely to be decimated than widespread taxa (**ALLENDORF and LUIKART, 2008; JEONG et al., 2012**). Importantly, the use of plants like *Cyrtanthus* species in traditional medicine involves the destructive harvesting of the whole plant. The often long growth cycles that characterises most of the *Cyrtanthus* species further reduces their chances of surviving the onslaught of modern day living. For example, **ISING** (**1970**) highlights that species such as *C. obliquus*, *C. herrei*, *C. carneus* and *C. huttoni*, among others, require between 5 to 15 years from seed to flowering. Even minor harvesting rates can be unsustainable for these species. Further to this, medicinal plant users believe that the most potent principles are located in the underground plant parts (**MANDER, 1998**), thereby encouraging non-sustainable harvesting from the wild. This pressure exerts ecological instability on the resource base, hence the need for conservation.

One of the central aspects of plant reproductive ecology is to determine the factors which govern plant fecundity (**JOHNSON and BOND, 1997**). The remarkable floral morphology and colour diversity in *Cyrtanthus* species has been identified with some reproductive complexities (**ISING, 1969; SNIJMAN and MEEROW, 2010**). Self-incompatibility is one of the mechanisms that characterises some of the species in the genera, a phenomenon associated with pollination uncertainty and far reaching ecological consequences (**ISING, 1969**). In a study on *C. breviflorus*, **VAUGHTON et al.** (**2010**) reports that the species is self-incompatible with late-acting self-rejection that allows both self- and cross-pollen tubes to penetrate the ovules. Because of the late acting nature of self-incompatibility in the species, and that pollinators deposit mixtures of both cross- and self-pollen, self-pollen disables ovules, reducing female fertility. Seed set of open-pollinated flowers thus become pollen-limited, despite pollen deposition exceeding ovule numbers (**VAUGHTON et al., 2010**). Species exhibiting this characteristics suffer reproductive costs under natural conditions and sustainability of their abundance and population viability under changing environments and other population decimating pressures is a challenge (**KNIGHT et al., 2005**). Being members of the Amaryllidaceae, *Cyrtanthus* are among species that exhibit extensive radiation in floral characters, paralleled with a wide diversity of specialised pollination systems (**JOHNSON and BOND, 1997**). Numerous studies undertaken in this regard conjures pollen limitation as a cause of floral radiation (**STEBBINS, 1970; ASHMAN et al., 2004; HARDER and JOHNSON, 2009**). A

direct and common ecological effect of pollen limitation is seed production. In a study on populations of *C. guthrieae* and *C. ventricosus*, fruit set and number of seeds increased more than 700% and 300% respectively in hand-pollinated flowers compared to natural fruit set (**JOHNSON and BOND, 1997**). Such a tremendous seed production increase following assisted pollination points to the fact that the two species are highly pollen limited. An inadequate reproductive efficiency resulting from pollen limitation means that the species' population sizes and viability are limited by seed production and would progressively decline if their utilisation rate in the wild is unmatched with conservative measures.

Another important aspect reported by **ISING** (**1969**) in *Cyrtanthus* species, is the numerical chromosome unbalance with a high frequency of structural chromosome changes that may result in altered karyotypes. Such chromosomal structural disturbances cause sterility in plants (**ISING, 1970**). Considering the fact that certain species of the genus like *C. ventricosus* only flower immediately following a fire, coupled with its floral reproductive limitation, their populations are at risk in the wild. In *C. guthrieae*, for example, **DUNCAN** (**2003**) highlights that its bulbs always seem to be solitary and never appear to reproduce by offset formation. *Cyrtanthus guthrieae* is one of the species characterised with floral reproductive complication and is reported to be rare and highly sought after for traditional medicine in the wild (**DUNCAN, 2003**). The flat, black papery seeds of most *Cyrtanthus* species takes three to four weeks to germinate with often erratic percentages. Taken collectively, the natural ecological limitations and human-driven consequences on the viability and population dynamics of *Cyrtanthus* in the wild is so extensive that alternative conservation measures has become an inevitable priority.

Due to the fact that in South Africa, most *Cyrtanthus* species used for horticultural and medicinal purposes are harvested from the wild their populations have been put under considerable strain. Approximately 2062 indigenous plant species (10% of the total flora) are recorded as being used for traditional medicine in South Africa, of which 82 species (0.4% of the total national flora) are threatened and a further 100 species are of conservation concern (**WILLIAMS et al., 2013**). The Amaryllidaceae family has about 42 species used in traditional medicine and 26.2% of these species are reported threatened or near threatened (NT). About 59 species in the entire

family are endangered or vulnerable and 58 species are near threatened (**SNIJMAN, 2004**). The National Red Data List places nearly half of the *Cyrtanthus* species as either, rare, vulnerable, critically endangered or their population is declining rapidly in the wild (**RAIMONDO et al., 2009; WILLIAMS et al., 2013**). In their list of South African medicinal plants that are threatened or of conservation concern, **WILLIAMS et al.** (**2013**) list *C. guthrieae, C. obliquus*, *C. suaveolens* and *C. mackenii* var *cooperi* as among these. A species is placed under the endangered category when the best available evidence indicates that it meets any of the five IUCN criteria for being endangered, and is therefore facing a very high risk of extinction in the wild. Factors that influence whether or not a taxa will face extinction threats include the size of the distribution range, the relative abundance of mature individuals in the wild and the intensity of the deterministic threat(s). Based on the market reports (**MANDER, 1998; DOLD and COCKS, 2002; VON AHLEFELDT et al., 2003; WILLIAMS et al., 2007; WILLIAMS et al., 2013**), the slow-growing bulbous plants are seemingly extracted at an unsustainable rate for medicinal purposes and highlights the importance of conservation management with respect to particular taxa

1.4. Propagation of *Cyrtanthus* species

1.4.1. Conventional propagation

Traditionally, *Cyrtanthus* species are either propagated through seed or bulblets. The balance between sexual and asexual reproduction vary markedly in these plant species. At one extreme, plants are sexually infertile and reproduction is solely clonal (**RAMSEY et al., 2011**). Bulbs often produce offsets that can be separated from the mother plant when they are large enough and planted to produce new plants. However, their multiplication rate is quite low in most species, with about two to five bulblets produced from one adult bulb per year (**MORÁN et al., 2003; HONG and LEE, 2012**). In some species like *C. breviflorus*, *C. guthrieae* and *C. staadensis*, the bulbs are solitary and do not reproduce vegetatively. Of the species that produce bulblets, the sizes formed naturally vary significantly and are not always uniform for use as propagules. The black, flat papery seeds of *Cyrtanthus* species have limited viability (**DUNCAN, 2002**). Germination of fresh seeds takes place within 4 weeks

from planting under ideal conditions. Amaryllidaceae plants have long generation times and can take up to 15 seasons to begin flowering (**DU PLESSIS and DUNCAN, 1989**). For this reason, propagation through conventional means becomes a challenge for the continued sustainable survival of these species in the wild.

1.4.2. *In vitro* propagation

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotent nature of plant cells to develop new plants. The technique has been employed extensively in the ornamental industry for large-scale plant multiplication of elite superior varieties (**ROUT et al., 2006**). The system can be employed for large-scale propagation of disease-free clones and gene pool conservation. Plant tissue culture technology offers an important alternative for rapid multiplication and germplasm conservation of rare and endangered plant species. The technology has an advantage over conventional propagation mechanisms in that it offers a defined system that ensures a continuous supply of planting stock with some degree of genetic uniformity (**FAY, 1992**).

The development of efficient and reproducible tissue culture and plant regeneration protocols have provided a foundation for the practical application of transgenic technology for improved plant breeding and the overcoming of incompatibility barriers, within an economically viable time frame (**HANSEN and WRIGHT, 1999**). In addition, plant tissue culture systems often serve as model systems in the study of physiological, biochemical, genetic and structural problems related to plants (**TORRES, 1989**). The genetic variability that results from somaclonal variation provides breeders with an excellent diversified gene pool to exploit in the development of superior varieties.

Micropropagation protocols have been developed for several of the Amaryllidaceae species to ensure rapid multiplication for horticultural and medicinal supply purposes as well as germplasm conservation (**McALISTER et al., 1998**). From the genus *Cyrtanthus*, very few of a total of about 60 species have established tissue culture

protocols in spite of the multitude of population decimation threats that confront many species of the genus in the wild (**JOHNSON and BOND, 1997; RAIMONDO et al., 2009; VAUGHTON et al., 2010**). The micropropagated species include, *C. brachyscyphus, C. breviflorus, C. elatus, C. falcatus, C. guthrieae, C. mackenii* (**McALISTER et al., 1998**), *C. clavatus, C. spiralis* (**MORÁN et al., 2003**), *C. loddigesianus* and *C. speciosus* (**ANGULO et al., 2003**). More research work on the tissue culture of these species needs to be conducted to establish protocols as a step towards their conservation.

1.5. Concepts in plant tissue culture/micropropagation

Although not different, in principle, from conventional asexual propagation of some plant species, the provision of aseptic and controlled environmental conditions, a heterotrophic development, and exogenous plant growth regulators (PGR) collectively contribute to the effectiveness of the micropropagation technique (**MURASHIGE, 1978)**. Competent cells within plant tissues respond to external signals and enter into specific developmental pathways (**MERCIER et al., 2003**). Regardless of the type and source of explant used, the pathway of regeneration undergoes several coordinated processes. An isolated tissue explant undergoes de-differentiation followed by re-differentiation and organisation into meristematic centres. Upon further induction the cells can form unipolar structures (organogenesis) or bipolar structures (somatic embryogenesis) (**ROUT et al., 2006**). In most systems examined, the organogenic process begins with changes in a single or a small group of parenchyma cells, which subsequently divide to produce a globular mass of cells or meristemoid, which can give rise to either a shoot or root primordium (**HICKS, 1980; THORPE, 1990; ROUT et al., 2006; GEORGE, 2008**). Organisation into morphogenetic patterns can either take place directly on the isolated explant or can be expressed only after callus formation, a process termed indirect morphogenesis (**GAJ, 2004**). These structural changes are interpreted as a manifestation of preceding physiological, biochemical, biophysical and molecular events which reflect selective gene activity in those cells (**THORPE, 1980; MURASHIGE and HUANG, 1985; BROWN and THORPE, 1986; THOMPSON and THORPE, 1990**)

In contrast to organogenesis, which produces a unipolar shoot or root primordium, somatic embryogenesis gives rise to a bipolar structure with a root/shoot axis. The two phased process involves the induction of cells with embryogenic competence, and their subsequent development into embryos (**THORPE, 1990; ZIMMERMAN, 1993**). Success in this regard is achieved through empirical manipulation of the explant, medium and culture environment in a very plastic process. The process up to the globular stage, begins with a quantal cell division in the presence of auxin (**NORMURA and KOMAMINE, 1986**). The resulting embryos may be structurally and/or cellularly distinct from zygotic embryos (**AMMIRATO, 1987; CONGER et al., 1987; THORPE, 1990**). In zygotic embryos, morphogenesis is regulated by two overlapping mechanisms, one of which arises as a maternal effect or as a consequence of the polarised position of the embryo in the embryo sac, while the other is intrinsic in the embryo itself (**WILLIAMS and MAHESWARAN, 1986; ZIMMERMAN, 1993**). The latter, intrinsic mechanism, which would logically be the only active mechanism in somatic embryos, is thought to be dependent on polar auxin transport. Although the existence of a dual system for auxin regulation of embryogenesis remains speculative, the similarity between zygotic and somatic embryogenesis is striking and remarkable, considering that somatic embryos develop completely outside both the physical constraints and the informational context of maternal tissue. (**ZIMMERMAN, 1993; VON ARNOLD et al., 2002; FEHER et al., 2003**). The role of exogenous auxin in somatic embryo induction also appears to depend on the type of the explant used. For example, hypocotyl explants (**KAMADA and HARADA, 1979**), petiole explants (**AMMIRATO, 1985**), and single cells isolated from established suspension cultures (**NOMURA and KOMAMINE, 1985**) required exposure to exogenous auxin for 1, 2, and 7 days respectively, before they were competent to undergo embryogenesis upon auxin removal.

1.5.1. Plant micropropagation- An overview

Micropropagation process generally involves five sequential but distinct stages: selection of mother plants, initiation of aseptic cultures, shoot multiplication, rooting of *in vitro* cultured shoots, and *ex vitro* acclimatisation (**MURASHIGE, 1978; DEBERGH and MAENE, 1981; KANE et al., 2008**). The first stage is concerned

with the selection of stock plants that could provide healthy explants. Healthy and vigorously growing plants are considered ideal for this purpose and at times preconditioning greenhouse growing treatments under optimal cultivation conditions is essential to increase the rate of explant survival (**DEBERGH and MAENE, 1981; KANE et al., 2008**). According to **HARTMANN et al.** (**1997**), the type of explant, where and how it is collected varies from species to species and with the experimental objective.

Virtually any plant organ/tissue can be used as an explant source but actively growing shoots are generally preferred for mass scale multiplication. The regenerative and morphogenic capacities of explants vary as a function of numerous and sometimes interacting factors. **MURASHIGE** (**1974**) highlighted the following as being key in the success of culture initiation: (i) the explant type; (ii) the physiological or ontogenic age of the donor plant at the time of excision and the degree of differentiation among constituent cells; (iii) the season in which the explant is obtained; (iv) the size of the explant; and (v) the overall quality and genotype of the stock plant. In addition to the above-mentioned guiding principles in making a good choice of explants, decontamination of the excised explants is of critical importance to the successful initiation and establishment of cultures (**TORRES, 1989**).

Once initiation and establishment of aseptic cultures has been successfully achieved, the third stage of micropropagation is concerned with optimising culture conditions for rapid shoot multiplication. Multiplication can be accomplished through either axillary shoot production, adventitious shoot proliferation or somatic embryogenesis (**MURASHIGE, 1978**). Axillary buds produce the smallest number of plantlets, as the number of shoots produced is limited by the number of axillary buds placed in each culture. Adventitious bud formation on the other hand has a greater potential for multiplication, as shoots may arise from any part of the explant. Somatic embryogenesis has the potential for producing the greatest number of plantlets, but has limitations that it can only be induced successfully in a limited number of species (**THORPE, 1980; ZIMMERMAN, 1993**). Although a particular predetermined regeneration pathway may be inherent within a specific tissue, the type and concentration of exogenously applied PGRs as well as culture conditions often modify the expression of this pathway.

In vitro grown plants are exposed to invariably controlled growth conditions such as high amounts of organic and inorganic nutrients, PGRs, carbon source, high humidity and low light intensities. Although these conditions may support rapid growth and multiplication, they tend to induce structural and physiological changes in plants rendering them unfit to survive when transferred directly to the field (**HAZARIKA, 2006; ROUT et al., 2006**). Prior to the transfer of elongated shoots, derived from the multiplication stage, to the outside environment, the objective of the fourth stage of the micropropagation process is to prepare plantlets for successful transfer to the soil (**MURASHIGE, 1978**). Rooting of plantlets is the first task at this stage and can either be accomplished *ex vitro* or *in vitro*. *In vitro* rooting is often achieved by culturing shoot cuttings on medium with half-strength concentrations of macro- and micronutrients, with or without auxins (**GABA, 2005; THORPE et al., 2008**). In some cases, the highest root induction occurs from excised shoots in liquid medium in contrast with the semi-solid medium (**ZIMMERMAN, 1993**). However, the response to rooting medium manipulations is often dependent on the plant species. For example, significantly higher rooting frequencies are reported in the shoots of some species cultured on full-strength medium in contrast to those cultured on half-strength (**WELANDER, 1983; REDDY et al., 2001; SUN et al., 2008**), while on the other hand, an inverse relationship between media strength and rooting frequency were noted in some species (**CHENG et al., 1992; PATIL, 1998; KOOI et al., 1999**). In cases where auxins are supplemented to induce rooting, the most frequently used auxins are, indoleacetic acid (IAA), indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) (**TORRES, 1989**). In some species rooting can be effected by other medium additives such as activated charcoal and phloroglucinol (**PAN and VAN STADEN, 1998**).

The heterotrophic mode of nutrition and poor mechanisms of controlling water loss render micropropagated plants vulnerable to transplantation shocks when transferred directly to a greenhouse or field conditions. Thus, a gradual acclimatisation process from *in vitro* culture to field condition is essential. A gradual shift from high humidity/low irradiance conditions to low humidity/high irradiance conditions, enables plantlets to survive under adverse environmental conditions (**ROUT et al., 2006**).

The success of micropropagation ultimately lies not only in the production of large numbers of plantlets but on their survival rate under field conditions. The fifth and final stage of micropropagation ensues that this objective is met. Successful acclimatisation procedures provide optimal conditions for higher survival rates and subsequent growth and establishment of micropropagated plants in the field. The physiological and anatomical characteristics of *in vitro* cultured plantlets necessitate that they be gradually acclimatised to the environment (**HAZARIKA, 2006**). Although specific acclimatisation details may differ, the most satisfactory techniques address the changes required for successful establishment and these include; lower relative humidity, higher light intensities, autotrophic growth and carbon dioxide enrichment, all of which are characteristic of greenhouse or field conditions (**TORRES, 1989; HAZARIKA, 2003; 2006**).

It must be clear, however, from the above description of the overview of micropropagation process that, although the stages of the technique may appear to be somewhat simple and straight forward, a multitude of interactive factors are involved and collectively determines its success and/or failure. The highly flexible nature of the ontogenic program of plants is linked to the reversibility of the differentiation state of somatic cells (**FEHER et al., 2003**) and this allows their developmental program to be much more open to alternative pathways. Under *in vitro* culture, the differentiated fate of plant cells are altered and the transient cell state induced by culture conditions is characterised by extensive cellular reorganisation that allows for a developmental switch, if appropriate signals are perceived. Among the factors that determines the direction and fate of cellular development *in vitro* are the chemical changes in the cellular environment (nutrient medium), culture environmental conditions and the physiology of the explant tissues (**MURASHIGE, 1974**). **FEHER et al.** (**2003**) pointed out that the formation and activity of meristems are highly dependent on drastic changes in environmental as well as developmental (PGRs) factors *in vitro*, which the authors collectively regarded as stress factors. Accordingly, the response of plant cells will thus depend on the level of stress and the physiological state of the cells. Of the physical factors, **MURASHIGE** (**1974**) notes that light and temperature are of major importance in plant tissue culture. Plant growth regulators on the other hand, are considered as the

most likely candidates in the regulation of developmental switches (**MURASHIGE, 1974; TORRES, 1989; FEHER et al. 2003**).

1.5.2. Plant growth regulators and plant tissue culture

Plants sense and respond to endogenous signals and environmental cues to ensure optimal growth and development. Plant cells integrate the myriad transduction events into a comprehensive network of signalling pathways and responses. Intercellular and interorgan communication ensues a coordinated development in plants and relies heavily on extracellular signalling molecules. Plant growth regulators occupy a central role within this transduction network to regulate plant physiological and developmental processes at micromolar or lower concentrations (**SWARUP et al., 2002; GEORGE et al., 2008; BAJGUZ and PIOTROWSKA, 2009**). Morphogenic responses of cultured tissues are typically described in relation to the type and concentration of PGRs in the medium. Auxins and cytokinins (CKs) are the main phytohormones involved in the regulation of cell division, elongation and differentiation. However, the developmental response of explants to exogenous PGRs is a result of a variety of biochemical processes, including their uptake, distribution and metabolism and subsequent effect on the endogenous PGR concentration in tissues (**CENTENO et al., 2003**). In addition to the fundamental understanding that intricate and spatiotemporal regulation of PGRs in developing plant organs primordia are achieved through transport and signal perception, genes crucial for biosynthesis of auxins and cytokinins reveal that localised biosynthesis also plays an important role in organ growth and patterning (**ZHAO, 2008**).

1.5.2.1. Cytokinins: structure metabolism and function

Cytokinins came to light when their ability to promote cell division in tobacco tissue cultures were discovered (**MILLER et al., 1955; SKOOG and MILLER, 1957**), and since then the N^6-substituted adenine-based molecules have been associated with numerous developmental roles (**ZHAO, 2008**). Being structurally diverse and biologically versatile compounds, the number of chemicals fitting the definition of CKs has grown to include a large array of natural, synthetic adenine and phenylurea derivatives (**MOK and MOK, 2001; SAKAKIBARA, 2006**). Naturally occurring CKs

are adenine derivatives carrying either an isoprene-derived or an aromatic side chain at the N^6 terminus and are conventionally classified as isoprenoid or aromatic CKs, respectively. In both groups, however, small variations exist in side-chain structure such as the absence or presence of hydroxyl groups and their stereoisomeric position (**SAKAKIBARA, 2006**). Isopentenyladenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (dZ) are by far the most prevalent CKs in higher plants (**MOUBAYIDIN et al., 2009**) (**Figure 1.2**). Among these, tZ and iP, including their sugar conjugates, are the major derivatives but lots of other variations exist depending on plant species, tissue, and developmental stage (**LETHAM and PALNI, 1983; SAKAKIBARA, 2006**). The best known CKs with ring substitutions at the N^6-position are kinetin and N^6-benzyladenine (BA). Modifications of the adenine ring on aromatic CKs mostly parallel those of the isoprenoid-types. Among the aromatic CKs, **STRNAD,** (**1997**) identifies *ortho*-topolin (oT), *meta*-topolin (mT), their methoxy-derivatives and BA to be only found in some plant species (**Figure 1.3**).

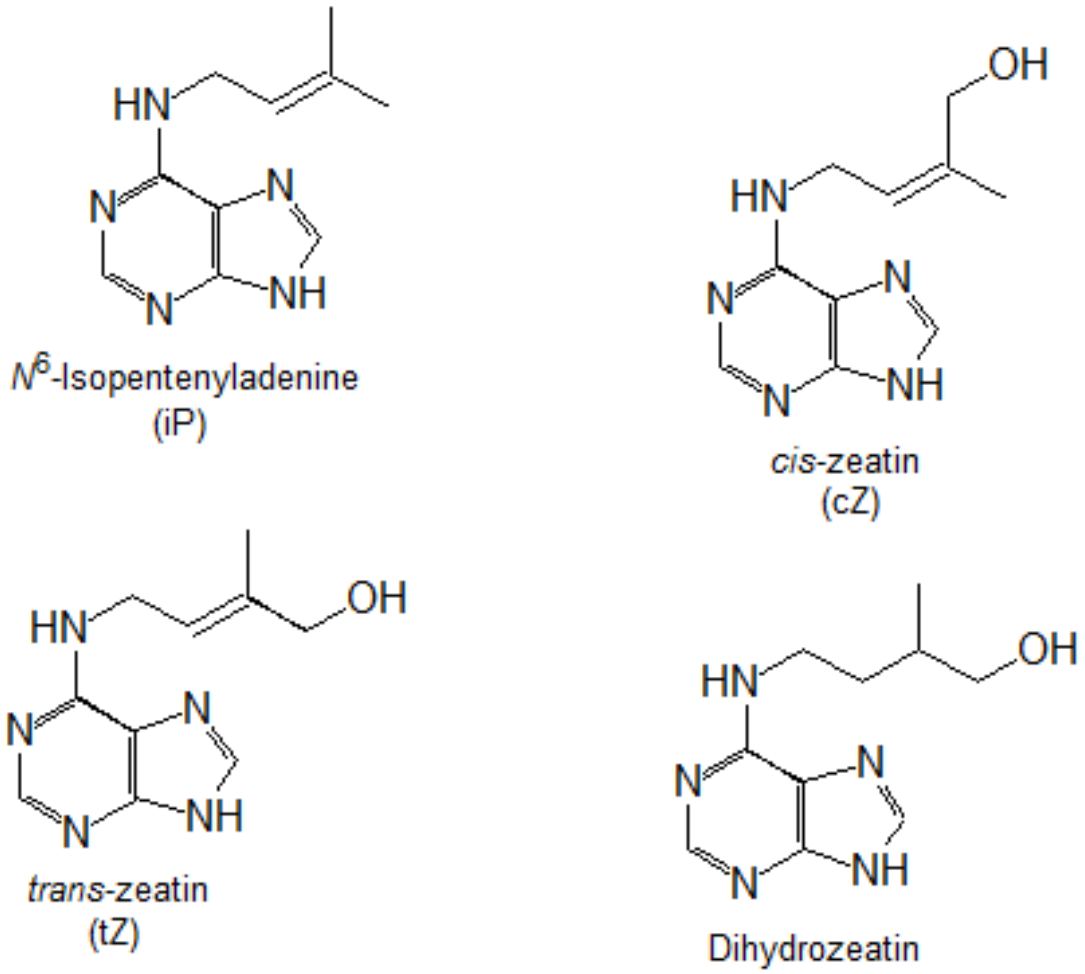


Figure 1.2. Chemical structures of some isoprenoid-type cytokinins commonly used in plant tissue culture

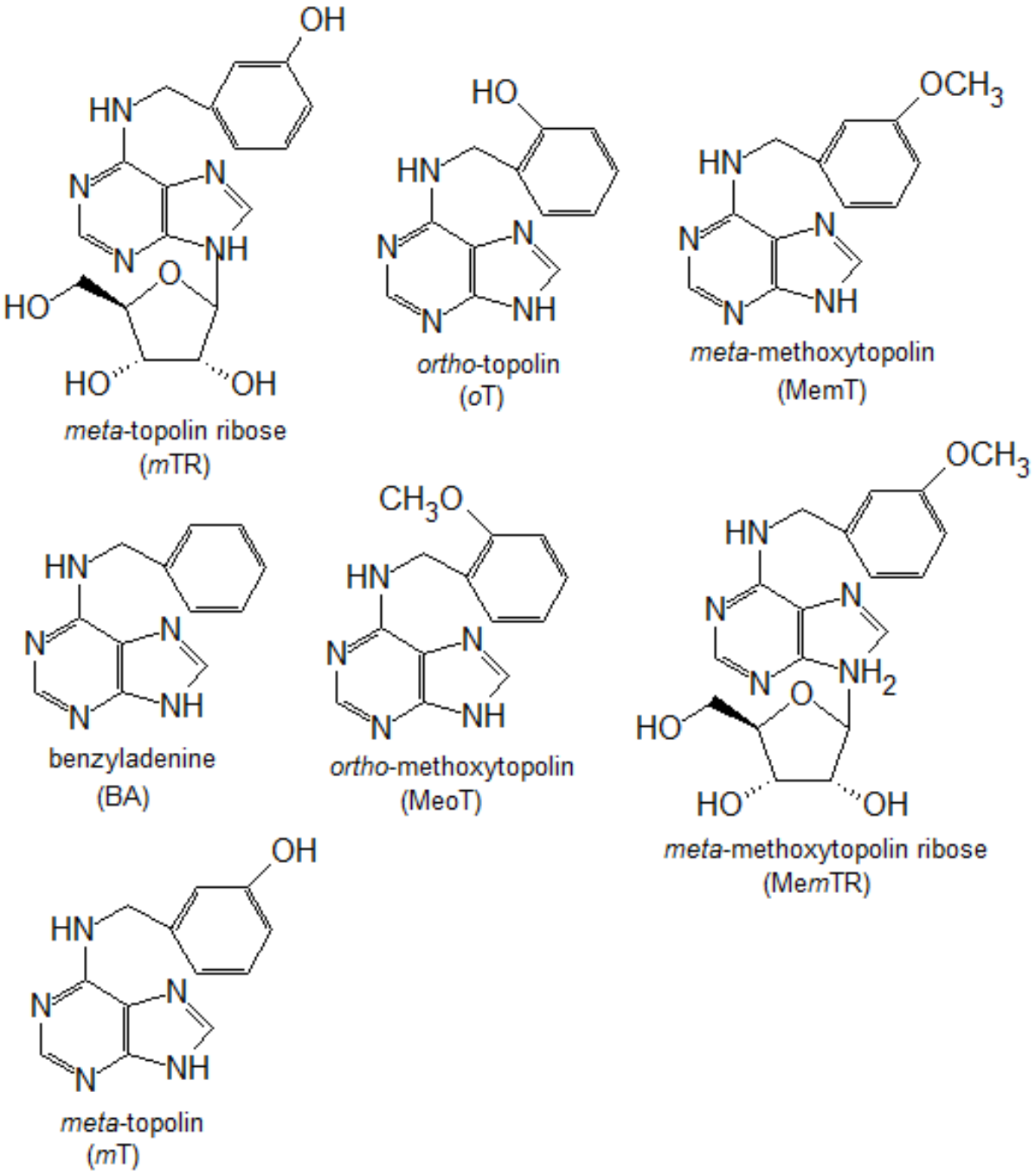


Figure 1.3. Chemical structures of some aromatic-type cytokinins utilised in plant tissue culture.

1.5.2.1.1. Biosynthesis and metabolism

The activity of CKs in plants is thought to be controlled by a fine balance between synthesis, catabolism and conjugation. Decades after CK discovery, genes required for their biosynthesis, degradation and perception, and their immediate downstream transcriptional effectors were brought to light (**SAKAKIBARA, 2006**). The rate-limiting step of CK biosynthesis is catalysed by the ATP/ADP-isopentenyltransferase (*AtIPT*) gene family, optionally followed by a cytochrome P450-dependent hydroxylation reaction, after which the sugar-phosphate moiety is cleaved off to yield the active cytokinin (**SAKAKIBARA, 2006; DOERNER, 2007**). Expression patterns of these genes indicate that CKs are produced at various sites in the plant, including roots, shoots and immature seeds (**MIYAWAKI et al., 2004; MOUBAYIDIN et al., 2009**). However, spatial expression patterns of *AtIPT*s through their promoter-reporter gene analyses, reveal tissue- and organ-specific patterns of CK synthesis (**MIYAWAKI et al., 2004**). Perception of CK is through transmembrane histidine kinase receptors (AHK2, AHK3 and AHK4/WOL1/CRE1) (**INOUE et al., 2001; HWANG and SHEEN, 2001; 2002**). The two-component signalling system is initiated when a histidine protein kinase sensor, modulated by an environmental cue, perceives a stimulus and autophosphorylates its own conserved histidine residue in the kinase domain and transfers the phosphate to a conserved aspartate residue of a response regulator (**HWANG and SHEEN, 2001; 2002; KAKIMOTO, 2003**) which activates downstream responses (**WEST and STOCK, 2001; TO and KIEBER, 2008**). The signal is then transmitted to the nucleus, to regulate the activity of genes encoding response regulators (ARRs) (**KAKIMOTO, 2003**). Type-B ARRs transcriptionally regulate the positive CK response whereas type-A ARRs are negative regulators of CK signalling. Thus, the proximal CK effector network is constructed with a negative feedback loop to control the magnitude of subsequent responses and this provides an elegant mechanism for other inputs to control cellular responses to CK (**LEIBFRIED et al., 2005; MOUBAYIDIN et al., 2009**). Metabolic inactivation of CK is under the influence of cytokinin oxidase/dehydrogenase genes, which catalyse the irreversible degradation of CKs (**SAKAKIBARA, 2006**).

The biosynthesis and cellular level balance of endogenous CK is fine-tuned by internal and external factors such as phytohormones and inorganic nitrogen sources, which appears important in linking nutrient signals and morphogenetic responses (**SAKAKIBARA, 2006**). The activity of CK oxidase degrades CKs and their ribonucleosides to adenine and adenosine, a key mechanism in controlling CK content in plant cells. Changes in the activity of CK oxidase detects changes in the CK concentrations in plant tissues and thus significantly contributes to the regulation of various cellular morphophysiological processes (**AVALBAEV et al., 2012**). The interconversion of CK bases, nucleosides and nucleotides to either metabolically active or inert forms is a major feature of CK metabolism in plant tissues (**CHEN, 1997**). The metabolic interconversion reactions are catalysed by a variety of purine metabolising enzymes such as glucosyltransferase, adenosine nucleosidase, and xylosyltransferase. The enzymes usually have higher affinity for adenine, adenosine, and AMP than for the corresponding cytokinins (**MOK and MOK, 2001**). Glucosyltransferase, catalysing the formation of CK glucosides at the 7- and 9-positions, recognises a range of adenine derivatives as substrates but the rate of conversion is highest for compounds with N^6-side chains of at least three alkyl carbons and correlates with CK activity (**ENTSCH et al., 1979**). Although the formation of 7-glucosides is favoured, the relative amounts of 7- and 9-glucosides formed differ between substrates, with ratios of about 2:1 for BA and 10:1 for zeatin (**MOK and MOK, 2001**). Natural CKs, therefore, exist as mixtures of free bases, nucleosides, nucleotides and other glycosides or amino acid conjugates in apparent equilibrium (**BAJGUZ and PIOTROWSKA, 2009**). Although these conjugates greatly reduce the biological activity of CKs, their biological stability is increased. Cytokinin specificity is determined by the structure of their side chains, and as such any slight modification can significantly affect the biological activity. Most bioassays, report the free base as usually the most active, and thus CK conjugation contributes to the regulation of their activity (**SCHMITZ et al., 1972; MATSUBARA, 1980; SCHMÜLLING, 2004**). Cytokinin conjugates seem to as serve storage, transport, and deactivation forms due to their resistant to degradation by CK oxidase/dehydrogenase (**AUER, 2002; BAJGUZ and PIOTROWSKA, 2009**), a phenomenon that presents another regulatory mechanism that allows physiological and developmental plasticity in plants.

1.5.2.1.2. Function and biological activity

Cytokinins play multiple roles in the modulation of plant growth and development. Together with auxins, CKs, take part in the regulation of the cell cycle in plant cells (**ZHAO, 2008**). Their ability to induce plant cell division is achieved by stimulating G1 to S and G2 to M transitions (**MOK and MOK, 2001**). Their involvement in G1-S regulation is supported by the observation that CKs increase G1 cyclin, cyclin D3 and that constitutive expression of cyclin D3 results in CK-independent growth of Arabidopsis calli (**KAKIMOTO, 2003**). The overexpressed calli were green compared to the wild-type, further suggesting the possibility that cyclin D3 may also regulate chloroplast development (**RIOU-KHAMLICHI et al., 1999**). In shoot apical meristems, cytokinin and cytokinin-related regulatory networks are necessary for maintenance of stem cells (**GALUSZKA et al., 2008**). Shoot apical meristem activity requires high CK levels, with the subsequent expression of at least two genes responsible for maintenance of stem cell position of the shoot apical meristem in undifferentiated cells, leading to a strong meristem phenotype (**TO et al., 2004; LEIBFRIED et al., 2005**). Inactivation of CK receptors leads to severe reduction in meristem size. Cytokinin production by meristem cells highlight a salient feature of meristem gene regulatory networks essential to coordinate growth and proliferation in adjacent cells and to maintain a homeostatically stable cellular state (**DOERNER, 2007**). Not only are CKs essential for meristem maintenance, but are also involved in establishing new meristems: for example, transgenic plants overexpressing genes for cytokinin oxidases, which are involved in CK degradation, have increased lateral root formation (**WERNER et al., 2003; LOHAR et al., 2004**). Through selective expression of a CK oxidase gene in different domains of the root meristem, the authors deduced that cytokinin levels specifically in the transition zone control meristem size, while reduced CK in the meristem has no effect on size.

Shoot meristems are responsible for all the post-embryonic aerial organs, such as leaves, stems and flowers and CKs are reported to play a positive role in the shoot meristem function (**KURAKAWA et al., 2007**). Ectopic expression of the rate-limiting factor (ARR2) in response to CK in transgenic Arabidopsis, was found to be sufficient to mimic CK in promoting shoot meristem proliferation and leaf differentiation, and in delaying leaf senescence (**HWANG and SHEEN, 2001;**

KURAKAWA et al., 2007). Fine-tuning of concentrations and spatial distribution of bioactive CK by CK-activating enzymes is one of the postulated mechanisms that regulates meristem activity (**KURAKAWA et al., 2007**). A severe reduction in the size of meristems in a mutant defective in all of its CK receptors provided compelling evidence that cytokinin is required for meristem activity (**WERNER et al., 2003; KURAKAWA et al., 2007**).

Cytokinins have a crucial role in the continuous division of both procambial and vascular cambium cells, processes which provide precursor cells for the xylem and phloem (**YE, 2002**). Mutation of the CK receptor genes leads to reductions in cell files and differentiation of all procambial cells into protoxylem, an indication that genes involved in cytokinin signalling are important regulators of vascular development (**MÄHÖNEN et al., 2000; HUTCHISON et al., 2006**). Mounting evidence also points to the complementary regulation between macronutrients and cytokinins for nutrient acquisition and distribution within the plant in response to environmental factors (**FRANCO-ZORRILLA et al., 2002; 2005; SAKAKIBARA, 2006; WERNER et al., 2006**). Auxin, cytokinin, and other unknown metabolites are thought to regulate apical dominance. Decapitation studies using pea plants led to increased levels of endogenous CKs in the stem and/or xylem sap, with a concurrent increased delivery of CKs to axillary buds. These lines of evidence suggest that cytokinins synthesised in the stem are transported to the axillary buds after decapitation, resulting in promotion of bud outgrowth (**LI et al., 1995; TANAKA et al., 2006**). The spatial expression patterns of CK metabolic genes and the unequal distribution of tZ- and iP type CKs in vascular transporting systems also emphasises that CKs act as both local and long-distance signals. Translocation of CK is purported to be mediated by subsets of purine permeases and nucleoside transporters by sharing the purine and the sugar conjugate transport systems, respectively (**HIROSE et al., 2008**).

The role of CKs has been inferred mostly from the effects of exogenous application. Although detailed mechanisms of action remain elusive and poorly understood, exogenously applied CKs in plant tissue culture, interact with the endogenous level and affect morphophysiological development changes through an altered balance in the cultured tissues (**BAJGUZ and PIOTROWSKA, 2009**). The resultant growth

and/or direction of morphogenic development of cultured cells thus depend on the uptake and metabolism of the applied CKs as dictated by numerous intrinsic and environmental cues (**KAMÍNEK et al., 2000**). Cytokinins promote cell division as well as stimulate shoot initiation and growth *in vitro*. In higher concentrations, CKs promote axillary shoot formation by opposing apical dominance regulated by auxin. They inhibit root formation and induce adventitious shoot formation.

1.5.2.2. Auxins: Structure

Auxin, identified as a PGR due to its ability to stimulate differential growth in response to gravity or light stimuli, belongs to chemically diverse compounds, most of which have an aromatic system such as indole, phenyl or naphthalene ring with a side chain containing a carboxyl group attached (**Figure 1.4**) (**BAJGUZ and PIOTROWSKA, 2009**). Naturally occurring members of this hormone group include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 4-chloro-indole-3-acetic acid (4-Cl-IAA), with IAA being the major form identified in plants (**COOKE et al., 2002**). Indole-3-acetic acid is a hetero-aromatic organic acid, consisting of an indole ring with a weak net positive charge and an acetic acid side chain. The acetic acid-indole bond at the third position of the indole ring is freely rotating, with the carboxyl group exhibiting a strong negative charge at neutral pH (**EDGERTON et al., 1994**). All natural auxins exist in plant tissues as both free acids and conjugated forms through ester or amide linkages. Ester conjugates of IBA dominate over its amide forms. Moreover, IBA conjugates are more easily hydrolysed and more slowly transported in different plant systems, perhaps leaving more PGRs at the plant base in comparison with conjugates of IAA. In addition, certain IBA conjugates are very active in bioassays (**LJUNG et al., 2002**). For the regulation of auxin homeostasis, various mechanisms exist, such as biosynthesis, degradation, transport, and conjugate formation.

1.5.2.2.1. Biosynthesis, transport and signalling

Within the entire plant structure and throughout the life cycle of a plant, auxin levels vary significantly, forming complex gradients that appear to be a central component of its regulatory activity for plant development (**BENFEY, 2002; BALUŠKA et al.,**

2005). Intricate networks with adaptive plasticity that integrate genetic and biochemical redundancy have evolved in plants to regulate auxin levels in specific tissues in response to changing environmental and developmental conditions. Despite its important roles in plant development, an elucidated auxin biosynthesis pathway(s) remains elusive and an understanding of these processes is still fragmented. Although plants share evolutionary conserved core mechanisms for auxin biosynthesis, different plant species may also have unique strategies and modifications to optimise their auxin biosynthesis (**ZHAO, 2010**).

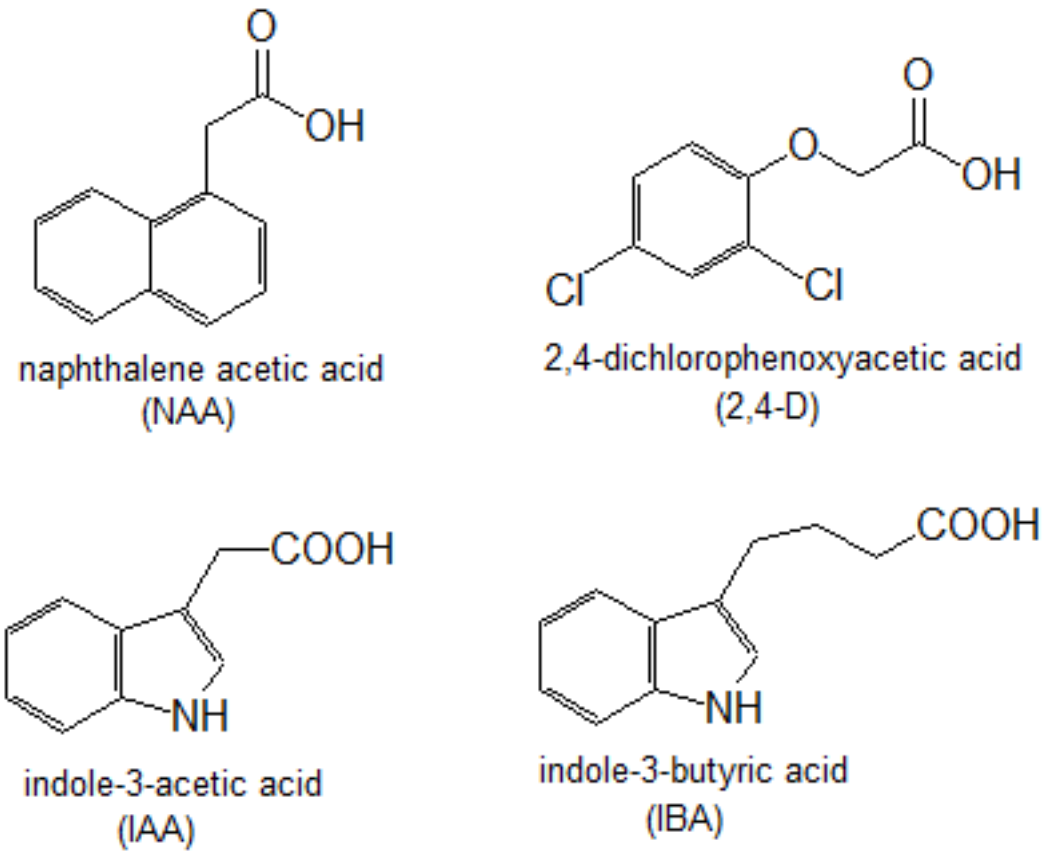


Figure 1.4. Chemical structures of commonly used auxins

Based on presumptive identified intermediates, genetic studies, and *in vitro* assays, a sketch of the auxin biosynthetic pathways has been modelled (**WOODWARD and BARTEL, 2005; VANNESTE and FRIML, 2009**). One tryptophan (Trp)-independent and four Trp-dependent pathways for the IAA biosynthesis have been proposed, each of which is designated for an intermediate key to the pathway (**WOODWARD and BARTEL, 2005**). These are the indole-3-acetamide (IAM) pathway, the indole-3-

acetaldoxime (IAOx) pathway, the tryptamine (TAM) pathway, and the indole-3-pyruvic acid (IPA) pathway. Although, plants employ several pathways to synthesise IAA, none of these is yet defined to the level of knowing each relevant gene, enzyme, and intermediate. Thus far, only the TAM and IPA pathways have been highlighted as relevant to development in plants (**WOODWARD and BARTEL, 2005; VANNESTE and FRIML, 2009**). Flavan monooxygenase-like enzymes of the YUCCA family are rate-limiting for the TAM pathway. The enzymes catalyse conversion of the Trp-derivative, TAM, to N-hydroxyltryptamine, a precursor of IAOx that is subsequently used in the biosynthesis of IAA (**MOCKAITIS and ESTELLE, 2008; ZHAO, 2010**).

The location of auxin biosynthesis appears to be an important aspect of overall regulation of auxin functions. *De novo* auxin production is highly localised and local auxin biosynthesis plays a key role in shaping local auxin gradients (**BALUŠKA et al., 2005; TAO et al., 2008**). Most auxins are synthesised in the shoot apex and distributed directionally and transcellularly towards the root apex within the stele. This transcellular as well as polar auxin transport is mediated by auxin influx and efflux facilitators of the PIN family, whose subcellular polar localisations guide the direction of auxin flow (**TANAKA et al., 2006**). PIN proteins are plant-specific transmembrane proteins with a predicted topology, suggestive of carrier proteins. The polar localisation of PIN auxin efflux carriers changes in response to developmental and external cues in order to channel auxin flow in a regulated manner for organised growth (**BENKOVÁ et al., 2003; SWARUP and BENNETT, 2003**). The expression and subcellular localisation of PIN proteins, is modulated by auxins themselves, contributing to a complex pattern of feedback regulation (**TANAKA et al., 2006**). Asymmetrically localised PIN proteins, represent a functionally redundant network for auxin distribution in both aerial and underground organs.

Asymmetric auxin distribution patterns and gradients have been observed within tissues and exhibit dynamic changes during different developmental processes. Recent findings demonstrate that auxin biosynthesis is regulated both temporally and spatially (**FRIML, 2003**). The expression of both YUC and TAA1 genes is restricted to a small group of discrete cells. For example, during embryogenesis both

YUC1 and TAA1 are initially expressed in the apical region at the globular stage, before gradual concentrations in the apical meristem at the heart stage, and finally restricted only to the apical meristem in the mature embryo (**CHENG et al., 2007; STEPANOVA et al., 2008; TAO et al., 2008**). Different combinations of YUC mutants yield different phenotypes, which often correlate with the gene expression patterns. Mutation of multiple YUC genes with overlapping expression domains impairs local auxin accumulation, leading to severe developmental defects in leaf venation, root pole specification, and floral organ patterning (**CHENG et al., 2006**). From these mutant studies, **CHENG et al.** (**2007**) suggested that auxin peaks are mainly generated from local synthesis rather than transport. Local auxin biosynthesis has also been shown to modulate gradient-directed planar polarity in root hair development in Arabidopsis (**TOBEÑA-SANTAMARIA et al., 2002; ZHAO, 2010**). It is, therefore, evident that both shoots and roots can produce auxins. YUC genes were also expressed in all organs including flowers, leaves, and roots in Arabidopsis, with each organ appearing to be self-sufficient in terms of controlling auxin gradients for development (**TOBEÑA-SANTAMARIA et al., 2002; IKEDA et al., 2009**). In *Arabidopsis thaliana*, planar polarity of root-hair positioning along epidermal cells is coordinated towards maximum concentration of an auxin gradient in the root tip (**GREBE, 2004; IKEDA et al., 2009**). Although computational modelling suggests that auxin efflux carrier activity may be sufficient to generate the gradient in the absence of auxin biosynthesis in the root, **IKEDA et al.** (**2009**) demonstrated that Raf-like kinase (CTR1) (**HUANG et al., 2003**) acts as a concentration-dependent repressor of a biosynthesis-dependent auxin gradient that modulates planar polarity in the root tip of Arabidopsis. The conclusion drawn, therefore, was that planar polarity relies on influx- and efflux-carrier-mediated auxin redistribution from a local biosynthesis maximum. Within an organ, YUC genes appear to be non-cell autonomous (**ZHAO, 2010**), allowing auxin synthesised by YUCs in one cell to be used by other cells. Thus, a local source of auxin biosynthesis contributes to gradient homeostasis during long-range coordination of cellular morphogenesis. Redundancy is, therefore, a key theme in auxin metabolism.

Subcellular positioning of auxin transporters determines the direction of auxin flow and provides a connection between signalling at the level of the individual cell and directional signalling within tissues (**VANNESTE and FRIML, 2009**). In this regard,

the mechanism provides each cell within the path of auxin flow with the option to integrate multiple signals and translate them into changes in expression and subcellular targeting of auxin transport proteins, thereby influencing how much auxin is to be transported and in which direction. Various environmental and endogenous signals can be integrated into changes in auxin distribution through their effects on local auxin biosynthesis and intercellular auxin transport. Thus, auxin appears to be a versatile trigger of pre-programmed developmental changes in plant cells and hence provides a means to efficiently integrate these signals. The interpretation of these auxin accumulations at the level of individual cells determines the type of developmental output (**VANNESTE and FRIML, 2009**).

An understanding of auxin biosynthesis provides a practical tool to manipulate auxin levels with temporal and spatial precisions, an exciting tool to tackle growth and development aspects particularly in plant tissue culture. Analyses of mutants defective in auxin signalling or transport are fundamental in elucidating the molecular mechanisms of auxin action in plant growth and development. Furthermore, the developmental defects due to insufficient and/or supra-optimal tissue levels of auxin in culture can be rescued by fine-tuned exogenous auxin treatments.

1.5.2.2.1. Function

The extremely wide spectrum of the plant processes that are influenced by auxin raises the question of how signals conveyed by a single molecule can trigger such a variety of responses (**BERLETH et al., 2004**). Auxins function as key regulators at the intersection of developmental and environmental events and the response pathways that they trigger. **DHARMASIRI and ESTELLE** (**2004**) describe auxins as implicated in virtually every aspect of plant growth and development, while **WEIJERS and JÜRGENS** (**2004**) liken the acronym for auxin, IAA to also stand for 'Influences Almost Anything'. Auxin regulates a plethora of physiological and developmental processes, including embryogenesis, vascular differentiation, organogenesis, trophic growth, root and shoot patterning, growth, branching and organogenesis, seedling development, fruit development, stress response, senescence, apical dominance and the patterned differentiation of cells in meristems and immature organs (**FRIML,**

2003; WEIJERS and JÜRGENS, 2004; QUINT and GRAY, 2006; GRUNEWALD and FRIML, 2010). At cellular level, auxin regulates these multiple responses by balancing cell division, elongation, and differentiation.

Despite the diversity of cellular responses evoked by auxin, most of the effects of auxin come down to one relatively simple pathway that directly impinges on transcriptional regulation (**VANNESTE and FRIML, 2009; ZHAO, 2010**). In essence, it is the interplay between two classes of transcriptional regulators that represents the core of auxin signalling. In most tissues, the auxin responses are concentration dependent and different tissues respond in a distinct manner to varying amounts of exogenous auxins (**LEYSER, 2001**). Auxins stimulate degradation of the Aux/IAA proteins, suggesting that auxins act, at least in part, by promoting the removal of these transcriptional repressors from the cell (**DHARMASIRI and ESTELLE, 2004**).

Polar placement and rapid relocalisation of auxin efflux carriers provide a conceptual basis for explaining changes in auxin flow in response to gravity and during root patterning. The polar transport of auxin is not only mechanistically involved in gravitropism (**BERLETH et al., 2004**) but also drives bending responses of organs in response to light stimuli, thus shaping the whole plant architecture (**COOKE et al., 2002; TANAKA et al., 2006**). Localised synthesis and/or polar transport establish IAA gradients that appear to be absolutely critical for positioning leaf primordia and floral meristems on shoot apices and for initiating lateral root primordia on mature roots (**GALWEILER et al., 1998; REINHARDT et al., 2000; CASIMIRO et al., 2001**).

Exogenously applied auxin in cultured tissues is rapidly taken up by plant cells through a chemiosmotic diffusion pH gradient (**BLAKESLEE et al., 2005**) and influx/efflux carriers (**VANNESTE and FRIML, 2009**), with subsequent conjugation to sugar, amino acid or inositol derivatives leading to their reversible inactivation (**ZHAO, 2010**). Upon hydrolysis, the conjugated forms yield biologically active free auxin, which can serve as transport and storage forms. The mechanism of action of exogenously applied auxins resembles that of endogenous ones in culture.

1.5.2.3. Auxin and cytokinin crosstalk

As a non-cell autonomous signal, auxin also interacts with other signalling pathways to regulate inter-cellular developmental processes. (**SWARUP et al., 2002**). Considering the multitude of processes affected by auxins, the phytohormone appears to be a master regulator of plant development and can be considered as a central organisation hub that monitors plant growth (**TANAKA et al., 2006**). Other phytohormones such as CKs agonistically or antagonistically feed into these auxin-dependent processes. This interdependent hormonal network (crosstalk) gets integrated at multiple levels, such as during auxin signalling, metabolism, and carrier-dependent auxin distribution (**NORDSTRÖM et al., 2004; IOIO et al., 2008; MOUBAYIDIN et al., 2009**). Auxin controls cytokinin biosynthesis (**NORDSTRÖM et al., 2004**) via the specific activation of *IPT5* and *IPT7* (**MIYAWAKI et al., 2004**) and most auxin-resistant mutants also show changes in their CK sensitivity (**NORDSTRÖM et al., 2004; IOIO et al., 2008**). The interactive relationship between auxin and CK, is often employed *in vitro* to induce root and shoot development, respectively. Auxin and cytokinin levels are inversely correlated *in vivo* and auxin treatment can rapidly inhibit cytokinin biosynthesis (**WOODWARD and BARTEL, 2005**). Physiological studies have established many links between the two groups of PGRs in regulating plant growth and developmental processes (**BAJGUZ and PIOTROWSKA, 2009**). The effects of auxin and cytokinin ratio on the morphogenic responses of cultured tissues were demonstrated by **SKOOG and MILLER (1957)**, and that has since served as the basis for plant tissue culture manipulation. The response of explants to PGR treatment vary considerably as a function of explant source, plant species and a balance between auxin and cytokinin (**VAN STADEN et al., 2008**). For example, when bulb explants were used, **PIERIK and STEEGMANS** (**1975**) and **FENNELL and VAN STADEN** (**2004**) reported that CKs were not essential for bulblet regeneration and growth in *Hyacinth* while *Narcissus* plantlets were produced in complete absence of exogenous PGRs (**HUSSEY, 1982**). **Figure 1.5** illustrates the general responses to various effects of auxin and CK interaction on *in vitro* cultures (**GEORGE and SHERRINGTON, 1984**). Although similar responses

cannot be demonstrated in all species, the concept has, however, drawn attention to the complexity of chemical interaction in the regulation of growth.

1.5.3. Environmental factors in plant microculture

One intriguing feature of *in vitro* culture, as opposed to any other production system, is the exacting degree with which the growth environment can be controlled. A plethora of both intrinsic and extrinsic factors interact to affect proliferation rate, morphogenesis and anatomy of plants grown *in vitro* (**MURASHIGE, 1974**). In addition to the composition of the medium, light (intensity, spectral quality and photoperiod), temperature and humidity are among the important environmental factors that play pivotal roles in the growth process of plants (**MURASHIGE, 1974; ANDERSON et al., 1995**).

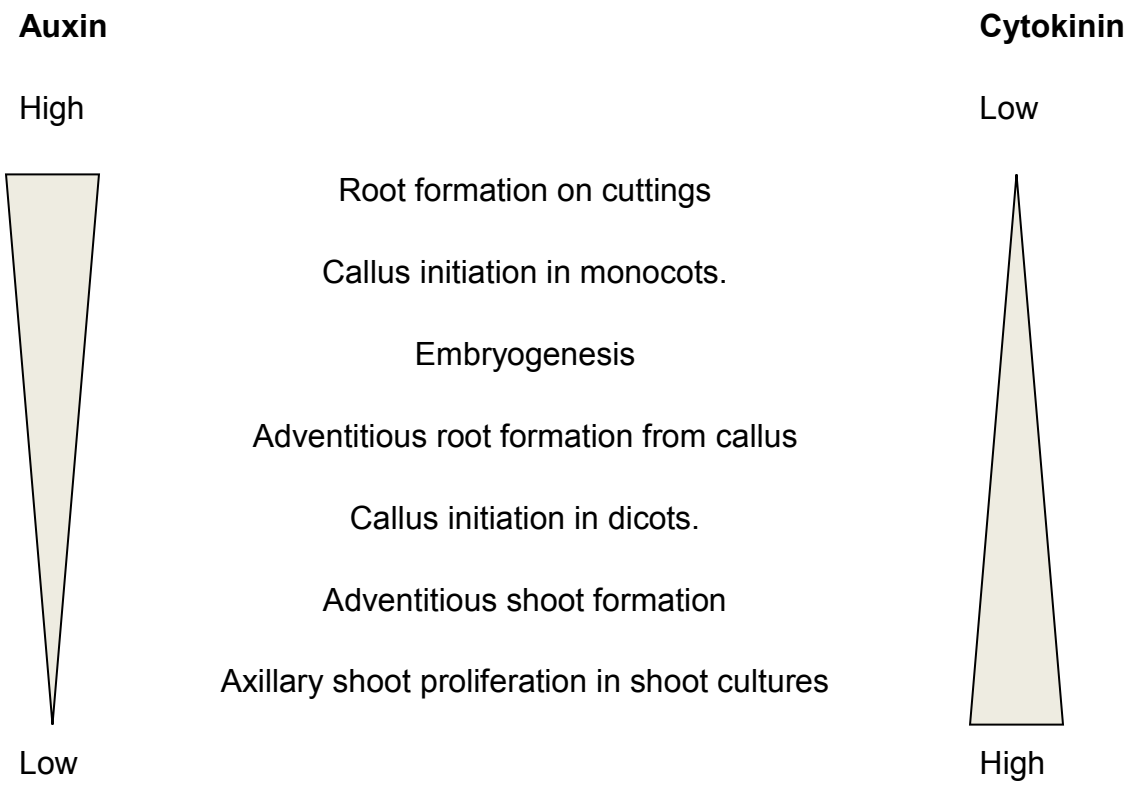


Figure 1.5. Effects of auxin and cytokinin ratio on the morphogenesis of plant *in vitro* cultures (**GEORGE and SHERRINGTON, 1984**)

1.5.3.1. Light

Photon energy drives photosynthesis required for plant growth and photomorphogenic processes (**ECONOMOU and READ, 1987**). Availability and quantity of light in natural environments vary spatially and temporary over several orders of magnitude and on a time scale that ranges from seconds to seasons. Due to the importance of light for plant growth, plants have evolved numerous biochemical and developmental responses to light that help to optimise photosynthesis and growth under fluctuating light regimes (**MIYAKE et al., 2009**). Although light requirements differ between species, the controlled light intensity for *in vitro* culture environment of between 40 and 80 µmol m^{-2} sec^{-1} (**HARTMANN et al., 1997**), is 15 times lower compared to 600-1 200 µmol m^{-2} sec^{-1} experienced in external growing environments. Generally, cool-white fluorescent lamps are used primarily for this purpose in most plant tissue culture systems as light sources (**KODYM and ZAPATA-ARIAS, 1998**).

Plant photosynthetic efficiency depends on the growth environment. Under microculture environments, the low light intensity, allows for “low photon energy” to be effectively absorbed by leaves and hence determine their photosynthesis rates (**MIYAKE et al., 2009**). Under these conditions, $NADP^+$ and ADP are rapidly regenerated, and the electrons flow efficiently in the photosynthetic electron transport. The photon energy absorbed by leaves in plants grown at high light intensity is usually higher than the rate of photosynthesis, i.e. photosynthesis becomes limited by the supply of CO_2 to chloroplasts through stomata (**VON CAEMMERER, 2000; MIYAKE et al., 2005**). Accumulation of electrons in the photosynthetic electron transport complex results in the photoinhibition of PSII, leading to a reduction in photosynthesis (**SÆBØ et al., 1995; MIYAKE et al., 2009**). For this reason, low light intensities in *in vitro* culture environments, therefore, provides an efficient flow of electrons in a linear electron flow, a characteristic favourable for the alleviation of PSII inactivation and thus an improved photosynthetic efficiency.

Despite the proposed improved photosynthetic efficiency of low light intensity *in vitro*, most findings report such plants as having reduced amounts and altered composition of cuticular waxes, less-developed palisade tissue and reduced stomatal function compared to outdoor-grown plants (**TERASHIMA et al., 2009; YAMANO et al., 2010**). In ultrastructural evaluations of *Liquidambar styraciflua*, **WETZSTEIN and SOMMER** (**1982**) reported *in vitro* plantlet leaves to have cells with large vacuoles, limited cytoplasmic content, and flattened chloroplasts with an irregularly arranged internal membrane system compared to field grown leaves. Photosynthetic rates of *in vitro* plantlets are generally low due to structural differences in the leaves, feedback inhibition from sugars in the medium and a restricted development of the photosynthetic apparatus (**WETZSTEIN and SOMMER, 1982; LEE et al., 1985; KODYM and ZAPATA-ARIAS, 1998**). The lack of internal chloroplast membrane development and lack of differentiation of a palisade parenchyma suggests that the photosynthetic capacity of these plantlets may be lower than that of naturally growing plants. The fact that *in vitro* cultured plantlets are grown on media supplemented with sufficiently large amounts of sucrose, allows provision for a total heterotrophic or photomixotrophic lifestyle (**LEE et al., 1985**). That being the case, the assumed limiting effects of low light intensities prevalent in most culture systems in terms of chloroplast differentiation and photosynthetic capacity becomes negligible and irrelevant.

Supporting this view, and contrary to most observations, **LEE et al.** (**1985**) reported tissue culture-differentiated leaves of *Liquidambar styraciflua* with well organised grana under low light intensity treatments, but disorganized at high light intensities. *In vitro* culture controlled photoperiodic conditions are reported to interact positively with PGRs by either activating CK biosynthesis or reducing its degradation, resulting in an increased endogenous CK level relative to that of auxin, with consequence morphogenic development in favour of this tilted balance (**KOZAI and KUBOTA, 2005**). The influence of photoperiod on the levels of endogenous PGRs to elicit different morphological responses in different plant species have been studied (**KUKLCZANKA et al., 1977; KAMADA et al. 1995; KOZAI et al., 1995; JO et al., 2008**).

Adventitious bud formation was reported from root explants of horseradish treated with auxin alone and cultured under a 16 h photoperiod (**KAMADA et al. 1995**). Stem diameter, leaf area and number of leaves in *in vitro* potato plantlets were significantly enhanced by long photoperiod and high photosynthetic photon flux (PPF) levels (**KOZAI et al., 1995**). In the same experiment, a long photoperiod and high PPF led to an increase in fresh and dry weights with an enhanced root growth. The authors concluded by suggesting that under photoautotrophic conditions a combination of high PPF level and long photoperiod produces potato plantlets *in vitro* of short and thick stem with a similar number of leaves and an increased leaf area, which are desirable for transfer to *ex vitro* conditions. In *Peperomia scandens*, **KUKLCZANKA et al.** (**1977**) reported improved shoot proliferation from leaf explants maintained under continuous light. **SÆBØ et al.** (**1995**) subjected *in vitro* cultures of *Betula pendula* to light of different spectral qualities, and reported highest photosynthetic capacity and chlorophyll content (2.2 mg dm^{-2} leaf area) and an enhanced leaf area in cultures irradiated with blue light (82 µmol dm^{-2} h^{-1}). Morphometric analysis of light micrographs has also shown that the epidermal cell areas and functional chloroplast area was largest in chloroplasts of plantlets subjected to blue light and smallest in those subjected to red light (**SÆBØ et al., 1995**). In their concluding remark, the authors suggested that light quality affects photosynthesis both through effects on the composition of the photosynthetic apparatus and on translocation of carbohydrates from chloroplasts. In embryo cultures of *Pseudotsuga menziesii*, red light enhanced adventitious bud formation, whereas blue light had no effect (**ECONOMOU and READ, 1987**). *Withania somnifera* plantlets grown under a photon flux density (PFD) of 30 mmol m^{-2} s^{-1} showed greater growth and development than those raised under other PFDs. Chlorophylls and carotenoids, numbers of stomata, photosynthetic and transpiration rates, stomatal conductance, and water use efficiency increased with increasing the PFD up to 60 mmol m^{-2} s^{-1} (**LEE et al., 2007**). Compared with the 8h photoperiod (short day), 16h photoperiods promote somatic embryogenesis in apical sections of the lateral roots of spinach seedlings (**MILOJEVIĆ et al., 2012**). One advantage of plant tissue culture systems is that the photoperiod, light intensity, and spectral quality can be manipulated to meet the required conditions for a particular objective.

1.5.3.1. Temperature

The biochemical reactions responsible for plant growth and development are driven by a diversity of enzymes at almost each integral step. For optimum performance, enzymes work effectively at specific temperature regimes. For this reason, *in vitro* cultures are often maintained at a constant temperature of approximately 25°C in most plant tissue culture settings, although temperature requirements may vary from species to species. The biophysical and biochemical process of photosynthesis is also temperature dependent (**TAIZ and ZEIGER, 2006**) and is pivotal in providing assimilates for growth and thus determines growth rates.

1.6. Plant secondary metabolites

Plants constantly interact with rapidly changing and potentially damaging external environmental factors. Being sessile organisms, plants have evolved elaborate intricate alternative defence strategies, which involve an enormous variety of chemical metabolites as tools to overcome stress conditions. Secondary metabolites play a major role in the adaptation of plants to the changing environment. Plants have an almost limitless ability to synthesise these metabolites. As a result of biotic and abiotic stresses, such as temperature, light intensity, herbivory and microbial attack, plants generate these defence mechanisms, triggering many complex biochemical processes (**HOLOPAINEN and GERSHENZON, 2010**). The synthesis of secondary metabolites is, however, often tightly regulated, and is commonly either restricted to specific plant tissues or developmental stages, or induced in response to stimulation factors (**OSBOURN et al., 2003; WINK, 2003**). As a strategy for survival and for the generation of diversity at the organism level, the ability to synthesise particular classes of secondary metabolites is also restricted to selected plant groups. Amaryllidaceae alkaloids, for example, are a group of nitrogenous secondary compounds exclusively limited in production to members of the Amaryllidaceae group of plant species. Although they are structurally diverse, secondary metabolites derive their synthesis from limited products of primary metabolism (**Figure 1.6**) (**CROZIER et al., 2006**). Intensive research efforts have elucidated the basic biochemistry and molecular biology of some biosynthetic pathways of secondary metabolism, with most of the findings supporting the

chemical reasoning that the diversification of secondary metabolism originates from the elaboration of a few central intermediates (**KUTCHAN and ZENK, 1993; KUTCHAN, 1995; FACCHINI, 1999; WINK, 2003**). Further studies reveal that their biosynthesis is not a random process, but rather highly ordered with respect to plant development, often exhibiting controlled expression of their pathways within organs, specific cells, and/or organelles within cells (**DE LUCA and ST PIERRE, 2000; OSBOURN et al., 2003**). Their synthesis depends on numerous enzymes involved in different metabolic pathways and their metabolism is completely integrated into morphological and biochemical regulatory patterns of plants. Phenolic compounds, alkaloids and terpenes are amongst the most commonly occurring secondary metabolites in the plant kingdom.

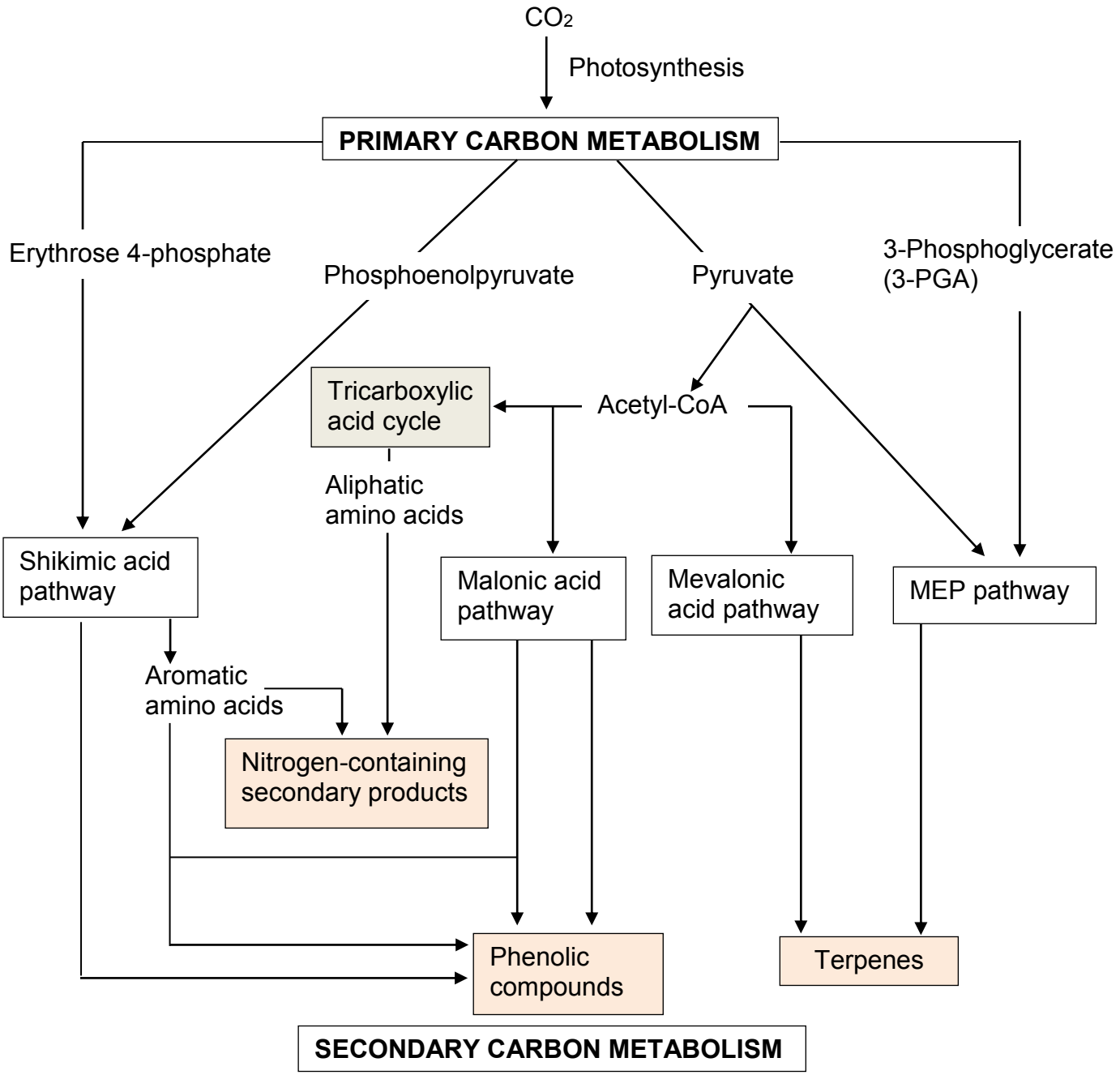


Figure 1.6. A simplified view of pathways involved in the biosynthesis of secondary metabolites (**TAIZ and ZEIGER, 2006**)

1.6.1. Alkaloids

Alkaloids encompass an enormous class of approximately 12 000 low-molecular weight natural products (**ZIEGLER and FACCHINI, 2008**). The principal requirement for classification as an alkaloid is the presence of a basic nitrogen atom at any position in the molecule, which does not include nitrogen in an amide or peptide bond (**FISCHBACH and CLARDY, 2007**). As implied by this exceptionally broad definition, alkaloids form a group of structurally diverse and biogenically unrelated molecules. As opposed to most types of secondary metabolites whose similar chemical structures are derived from related biosynthetic pathways, the many classes of alkaloids have unique biosynthetic origins (**FACCHINI, 1999**). Alkaloid biosynthesis and accumulation are associated with a variety of cell types in different plants, including epidermis, endodermis, pericycle, phloem parenchyma, phloem sieve elements and companion cells, specialized mesophyll, and laticifers. A common paradigm is the involvement of multiple cell types and the implied transport of pathway intermediates and/or products (**ZIEGLER and FACCHINI, 2008**). The complex intracellular compartmentation of alkaloid biosynthesis is thought to have occurred as a consequence of adapting compartmented reactions of primary metabolism to participate in alkaloid biosynthesis (**DE LUCA and ST PIERRE, 2000**). The subcellular trafficking of pathway intermediates also creates an important level of metabolic regulation that could not occur if enzymes and substrates diffused freely in the cytosol. Alkaloids are commonly synthesised from amino acids as starting precursor molecules, although some purine-derived alkaloids are also known. The substrate starting material typically define the structural class of the alkaloid (**FACCHINI, 1999**).

1.6.1.1. Amaryllidaceae alkaloids: Biosynthesis and biological activity

The Amaryllidaceae alkaloids, represent a group of isoquinoline alkaloids, which are produced almost exclusively by members of the Amaryllidaceae family. Although there are several other alkaloids having structures derived from these main molecular frameworks, Amaryllidaceae alkaloids may be classified into nine skeletally homogenous subgroups. Representative alkaloids from each of these

classes include norbelladine, lycorine, homolycorine, crinine, ismine, tazettine, narciclasine, montanine, and galanthamine (**MARTIN, 1987**) (**Figure 1.7**).

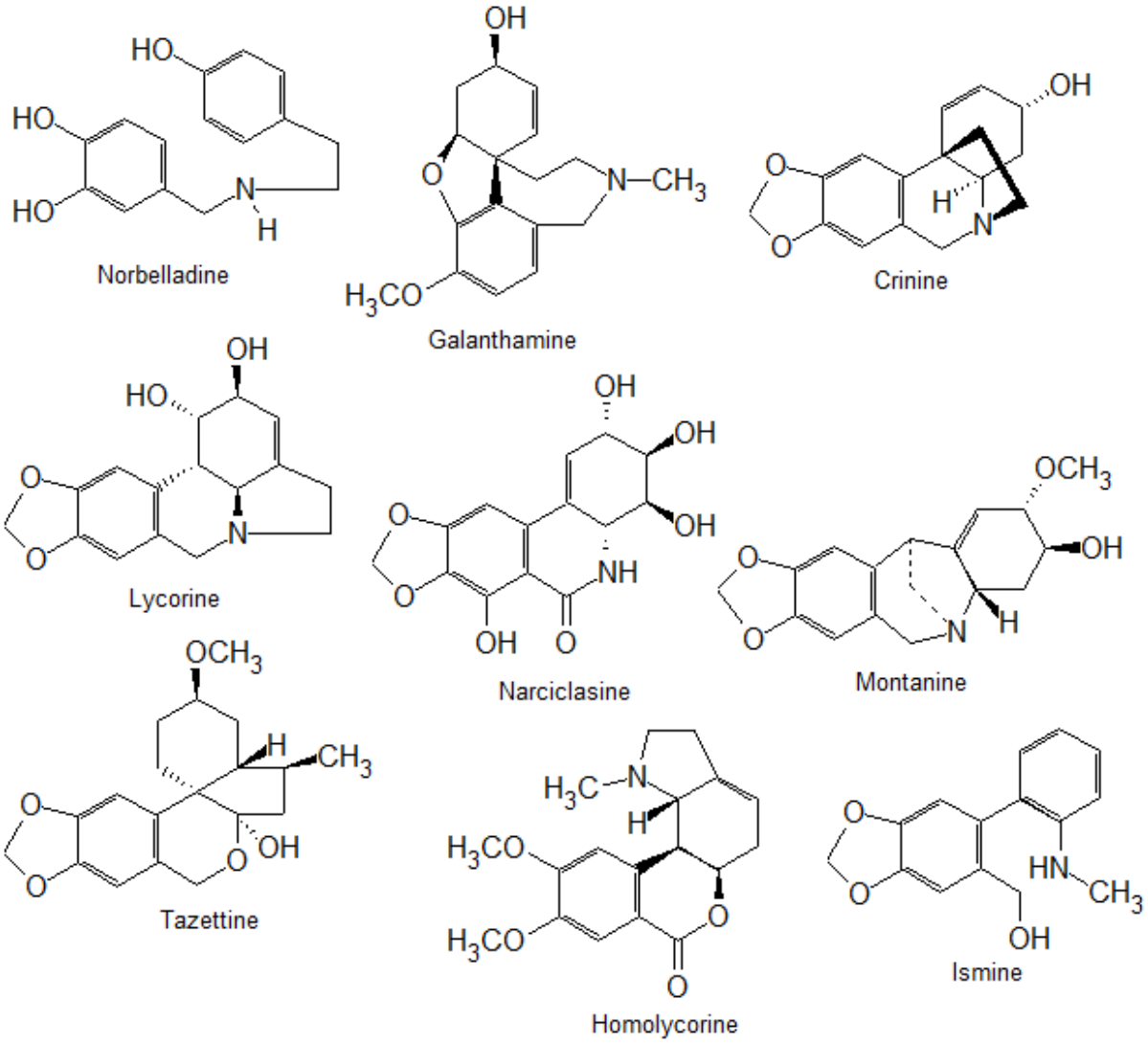


Figure 1.7. Representative members of the diverse structural classes of the Amaryllidaceae alkaloids derived from the common biosynthetic precursor norbelladine

Although the precise chemistry and the nature of enzymes involved in the early steps of isoquinoline alkaloid biosynthesis are less well characterized, the compounds are known to be formed biogenetically by intramolecular oxidative coupling of norbelladine and its derivatives (**BENTLEY, 1998; UNVER, 2007**). The biosynthetic processes is initiated by the formation of norbelladine (**FACCHINI and DE LUCA, 1995**), which later undergoes oxidative phenolic coupling, and subsequent transformation into the final alkaloid structures (**DE LUCA and ST PIERRE, 2000**).

Phenylalanine and tyrosine are the precursors of norbelladine. Key regulatory functions are often associated with enzymes, such as phenylalanine ammonia-lyase (PAL) that operate at the interface of primary and secondary metabolism (**CHOU and KUTCHAN, 1998**). Decarboxylation of these amino acids is an important step in the biosynthetic pathway of these group of alkaloids. Oxidative phenol couplings, which give rise to new skeletons, is responsible for the wide structural diversity in the isoquinoline group of alkaloids.

Although the exact roles of many alkaloids are not well understood, the compounds are believed to play an important ecological role, enabling the producing organism to interact defensively with its environment. They confer a survival benefit through their ability to bind to cellular targets in competing organisms (**MAPLESTONE et al., 1992; FACCHINI, 2001**). Due to the toxic nature of most alkaloids, their biosynthesis provides a general defensive mechanism for the producing organism. One such example is caffeine which has been demonstrated to act as a natural insecticide in plants. When the three *N*-methyltransferase genes involved in caffeine biosynthesis were overexpressed in tobacco, the resulting increase in caffeine production improved the tolerance of the plants to certain pests (**KIM et al., 2006; KIM and SANO, 2008**). The pharmacological uses of many alkaloids are well defined. These include, the potent inhibition of acetylcholinesterase (with potential application to Alzheimer's disease therapies), cytotoxicity, antibacterial, antiviral, anti-inflammatory, antiparasitic, antihistaminic, antiproliferative and adrenergic activity, antineoplastic against murine P-388 lymphocytic leukemia, treatment of mental disorders and age-related dementia (**VILADOMAT et al., 1997; HOUGHTON et al., 2006; MCNULTY et al., 2011; NAIR and VAN STADEN, 2013**). The wide chemical diversity and the limitless ability of the Amaryllidaceae plants to produce these alkaloids represent an extremely rich biogenic resource for the discovery of novel and innovative drugs.

1.6.2. Phenolic compounds

Phenolic compounds or polyphenols are characterised by at least one aromatic ring bearing one or more hydroxyl substituents, and other functional derivatives such as esters, methyl esters and glycosides (**DEY and HARBORNE, 1989**). They are a diverse group of higher secondary metabolites, with derivatives of the pentose

phosphate, shikimate pathway and phenylpropanoid metabolism, with phenylalanine as the precursor molecule (**RYAN et al., 1999; BALASUNDRAM et al., 2006**). The phenylpropanoid pathway is one of the most important metabolic pathways in plants in terms of carbon flux, with more than 20% of the cell total metabolism going through this pathway (**DIXON and PAIVA, 1995**). From a polyphenol point of view, the pathway yields, among others, flavonoids, lignans, lignins and anthocyanins. Key to the biosynthesis is the enzyme PAL which converts phenylalanine into *trans*-cinnamic acid by a non-oxidative deamination process. The enzyme plays an important role in controlling the flux into the pathways. The basic building unit of polyphenol is phenol. Although polyphenols are ubiquitous in the plant kingdom, the type of compound produced varies considerably between genera and species (**CROZIER at al., 2006**). Phenolic compounds form an integral part of the cell wall structure in plants, mainly in the form of polymeric materials such as lignins which serve as mechanical support and barriers against microbial invasion (**WALLACE and FRY, 1994**). The compounds exhibit a considerable free radical scavenging activity, determined largely by their reactivity as hydrogen- or electron donating agents and the stability of the resulting antioxidant-derived radical, which prevent the oxidation of various food ingredients, particularly fatty acids and oils (**RICE-EVANS et al., 1997; SUBBA RAO and MURALIKRISHNA, 2002**). Flavonoids and tannins are amongst the broad groups of phenolic compounds

1.6.2.1. Flavonoids: Biosynthesis and biological activity

Built upon a flavone skeleton, flavonoids are the most widespread and diverse class of low molecular weight phenolic compounds (**HEIM et al., 2002**). Biosynthetically, they are derived from a combination of the shikimic acid and the acetate pathways (**WATERMAN and MOLE, 1994**). They are constituents of a variety of plant parts, including, leaves, fruits, seeds, flowers, and roots, with over 4 000 different variants identified in plants (**HEIM et al., 2002; CROZIER at al., 2006**). They are found in high concentrations in the epidermis of leaves and the peels of fruits (**HRAZDINA, 1992; CROZIER at al., 2006**). Small differences in basic substitution patterns give rise to several subgroups, with the main subclasses being flavonols, flavones, flava-3-ols, isoflavones, flavanones and anthocyanidins (**HEIM et al., 2002**). The generic flavone structure is as illustrated in **Figure 1.8**.

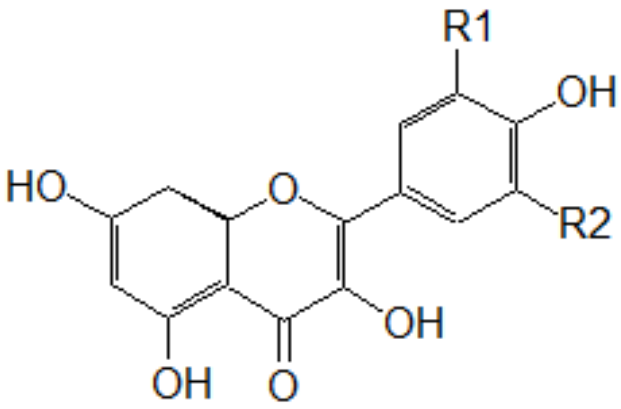


Figure 1.8. Generic structure of a flavonoid. Kaempferol, R1=H, R2=H; quercetin, R1=OH, R2=H; myricetin, R1=OH, R2=OH

Structural and chemical diversity of flavonoids is related to their diverse properties and roles in plants. In the producing plants, flavonoids provides protection against ultraviolet radiation, invading pathogens, and herbivores (**HARBORNE and WILLIAMS, 2000**). **HRAZDINA** (**1992**), working with a *Pisum sativum* cv. *argenteum* mutant, reported the highest accumulation of both anthocyanin and flavonol glycosides in the upper epidermal layers of leaf peels, which were directly exposed to visible and ultraviolet (UV) light irradiation, confirming a UV protection role for these compounds. In view of this, environmental factors seem to have a significant contribution to the content of flavonoids and phenolic acids in plants. The anthocyanin co-pigments in flowers serve to attract pollinating insects (**HARBORNE and WILLIAMS, 2000**) and are responsible for the characteristic red and blue colours of berries, wines, and certain vegetables all of which are major sources of flavonoids in the human diet (**CARANDO et al., 1999; STEWART et al., 2000; LOPEZ et al., 2001**).

One of the ascertained functions of flavonoids in plants is their protective role against microbial invasion (**WATERMAN and MOLE, 1994; HARBORNE and WILLIAMS, 2000**). This is achieved through their presence in plants as constitutive agents and also their accumulation as phytoalexins in response to microbial attack (**GRAYER et al., 1994; HARBORNE, 1999**). The ability of flavonoids to inhibit spore germination of pathogens has been explored and exploited in both traditional and modern human

medicine for the treatment of human pathogenic diseases. This is associated with the long history of the use of plants as medicinal remedies.

Flavonoids have attracted a lot of research interest in the pharmacological and pharmaceutical spheres, and this has seen characterisation of various plant flavonoids as antifungal, antibacterial, antiviral, anti-inflammatory, antioxidant, antitumor, anti-hepatotoxic, anti-lipolytic, vasodilator, immunostimulant and, antiallergic agents (**IINUMA et al., 1994; ALIAS et al., 1995; WILLIAMS et al., 1999; BURDA and OLESZEK, 2001**). Several consistent lines of evidence support the role of flavonoids in radical scavenging, chelating and oxidant activities against various reactive oxygen species (ROS) in animal cells. Consistent with most polyphenolic antioxidants, both the configuration and total number of hydroxyl groups, in addition to the flavan backbone, substantially influence several mechanisms of antioxidant activity and thus the free radical scavenging capacity of flavonoids can primarily be attributed to this characteristic (**HEIM et al., 2002; MIDDLETON et al., 2008**).

The HIV epidemic has stimulated an ever increasing interest in plant flavonoids as potential sources of drug agents for controlling the immunodeficiency virus, a causative agent of AIDS (**HARBORNE and WILLIAMS, 2000**). It is clear that plant flavonoids are playing a vital role in human health systems and in the drug discovery process.

1.6.2.2. Tannins: Biosynthesis and biological activity

Tannins are phenolic compounds that exhibit complex and highly variable chemical structures. They are broadly categorised into hydrolysable and condensed tannins, based on whether acids or enzymes can hydrolyse the components or whether they condense the components to polymers (**SCHOFIELD et al., 2001**). Both classes of tannins are rich in highly reactive hydroxyl groups which emanate from each of the benzene rings, and form complexes with proteins, including enzymes (**WALLACE and FRY, 1994**), and polymers such as cellulose and hemicellulose (**HASLAM, 1989**).

Hydrolysable tannins are based upon the fundamental structural unit of gallic acid (3, 4, 5-trihydroxy benzoic acid) and are almost invariably found as multiple esters with D-glucose to form gallotannins (**HASLAM, 1989**). Derivatives of hexa-hydroxydiphenic acid (ellagitannins) are derived from oxidative coupling of adjacent galloyl ester groups in a polygalloyl D-glucose ester (**MUELLER-HARVEY, 2001**). The central compound, pentagalloylglucose, is the starting point for many tannin structures. The compound consists of polyols, such as glucose, surrounded by several gallic acid units. Gallic acid and its metabolites are widely distributed in plants and particularly herbaceous dicotyledons (**MUELLER-HARVEY, 2001**). In particular plant tissues, e.g. plant galls, metabolites of gallic acid often accumulate in substantial quantities and constitute a reagent of considerable antiquity used in the analysis of mineral water and as a component of invisible ink (**HASLAM, 1989**).

Condensed tannins, commonly referred to as proanthocyanidins, are oligomers of 3-flavanols (catechins) and 3,4-flavan-diols (leucoanthocyanidins) linked together by single interflavan carbon to carbon bonds (**HAGERMAN, 2002; XIE and DIXON, 2005**). The flavan-3-ol units are linked principally through the 4 and the 8 positions. The term proanthocyanidin is derived from the acid-catalysed oxidation reaction that produces red anthocyanidins upon heating in an acidic alcohol solution (**PORTER et al., 1985**), a reaction that forms the basis of the butanol-HCl assay for proanthocyanidins. Their structures depend upon the nature (stereochemistry and hydroxylation pattern) of the flavan-3-ol starter and extension units, the position and stereochemistry of the linkage to the ‘‘lower’’ unit, the degree of polymerisation, and the presence or absence of modifications such as esterification of the 3-hydroxyl group (**Figure 1.9**). Most proanthocyanidins are built from the flavan-3-ols, catechin and epicatechin skeletons (**XIE and DIXON, 2005**). They are the most widespread polyphenols in plants after lignins and can be found in leaves, fruits, woods, barks, or roots, often in high concentrations (**MATTHEWS et al., 1997**). In plants, condensed tannins may act as feeding deterrents in reproductive tissues and developing fruit and also impart astringency to fresh fruit, fruit juices and wine (**HASLAM, 1989**). **FEENY** (**1970**), surmised that tannins, are characteristic of the chemical defence of plants and act as quantitative-dosage dependent-barriers to predators that may feed on them. The relevant physiological effects of tannins upon predation is assumed to be derived from their ability to complex with proteinaceous materials.

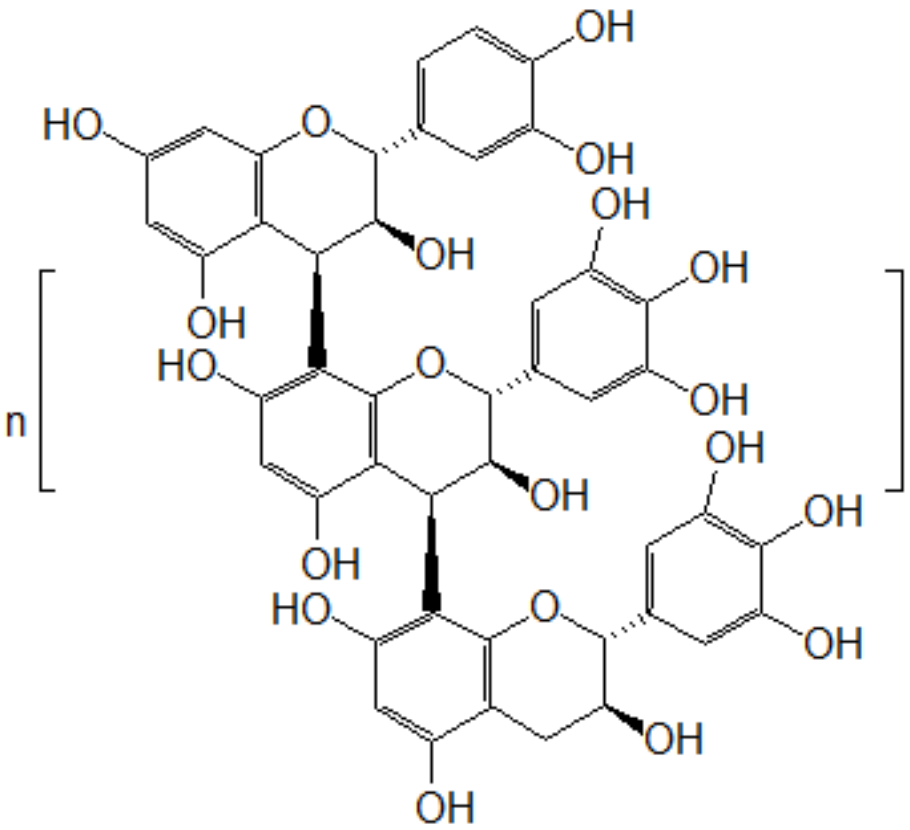


Figure 1.9. Chemical structure of a proanthocyanidin consisting of the catechin and epicatechin polymeric units, where 'n' is any number that makes up the polymer

Due to their antibiotic, antifeedant, or biostatic effects on a variety of organisms that consume them (**HASLAM, 1989**), the chemical properties of both condensed and hydrolysable tannins have been exploited in the discovery of versatile medicinal agents. Tannins are thus, reported to possess numerous medicinal properties such as antibacterial, antifungal, antiviral, anti-diarrhoeal, free radical scavenging, immunomodulatory, anti-inflammatory, anti-tumour and antidote activities (**OKUDA et al., 1992; HASLAM, 1996; GURIB-FAKIM, 2006**). Some, and certainly most of the beneficial effects which tannins exert as constituents of drugs and herbal remedies may well follow from their interaction with enzymes (proteins) within cell systems (**SCHULTZ et al., 1992; HASLAM, 1996**). **VANDEN BERGHE et al.** (**1985**) evaluated the claims for antiviral activity made for natural products, including various tannins, derived from over 900 plant species, and concluded that tannins act principally by binding to the virus and/or protein of the host cell membrane and thus

arrest adsorption of the virus. Similarly, bacterial and fungal enzymes and toxic proteins may be bound by tannins and inactivated in a similar manner (**SCHULTZ et al., 1992**).

1.7. Environmental factors on plant secondary metabolites

The most common, inevitable interaction occurring in plant communities is the plant-environment interaction. Throughout the course of growth and development, plants are exposed to various biotic and abiotic factors to which they respond with an activation of their defence system. External factors quantitatively affect the plant's metabolic processes through their effects on plant development, growth rates and partitioning of assimilates into vital metabolites. Despite their lack of mobility and the multitude and extensive range of stress factors encountered in their natural habitats, plants are, however, able to exploit any ecological niche. Paralleled with this intricate environmental interaction is an equally elaborate, intrinsically evolved morphological, physiological and biochemical plasticity in plants that ensures survival (**DE LUCA and ST PIERRE, 2000**). Cellular organisation is one of the defining characteristics in this phenomenon, with secondary metabolism forming an integral component of this adaptive mechanism. A great deal of scientific evidence, implicate the highly ordered interactions between plants and their biotic and abiotic environments to be the major driving force behind the emergence of specific secondary metabolites (**FACCHINI, 1999; VASQUEZ-ROBINET et al., 2008; SPERDOULI and MOUSTAKAS, 2012a**). Despite the complex nature and inconclusive reports on the regulatory mechanisms and biosynthesis of different secondary metabolites in plants, a significant body of research evidence posits their biosynthesis and accumulation to be remarkably under the influence of the environment (**SZATHMÁRY et al., 2001; HOLOPAINEN and GERSHENZON, 2010**). A number of secondary metabolites have established ecophysiological and defence adaptive roles.

Drought, salinity, temperature and soil nutritional status are among the major abiotic factors affecting plant growth and are reported to trigger abrupt activation of qualitative and quantitative changes in cellular secondary metabolite pools (**LAUGHLIN, 1993; PÉREZ-ESTRADA et al., 2000; LOMMEN et al., 2008**).

1.7.1. Osmotic and salinity stress

Moisture and salt stress induces molecular damage in plant cells leading to hyperosmotic stress, homeostatic disruption, and ionic toxicity. Mild effects of the two stress factors are primarily limited to plant growth and development, while in extreme cases, plant mortality is often the result (**SOBHANIAN et al., 2011**). The key primary process of plant growth, photosynthesis, is directly and indirectly affected by osmotic constraints leading to a reduction in carbon assimilation (**CHAVES et al., 2009**). The effects of osmotic and salinity stress on photosynthesis range from the restriction on CO_2 diffusion into the chloroplast, via limitations on stomatal opening mediated by shoot- and root-generated hormones, and on the mesophyll transport of CO_2, to alterations in leaf photochemistry and carbon metabolism (**GALME´S et al., 2007; CHAVES et al., 2009**). Exposure of chloroplasts to excess excitation energy limits carbon dioxide fixation, leading to the generation of reactive oxygen species (ROS) and subsequently introduces oxidative stress (**FEHER et al., 2003**). In the case of salinity stress, in addition to cellular osmotic constraints, plants endure salt-specific effects. Salt response follows a biphasic model, with current metabolic evidence indicating an early similarity with drought, while in the long-term, plants deal with ion toxicity (**CHAVES et al., 2009**).

The intensity, duration and rate of progression of the stress factor influence a plant's response to water scarcity and salinity. The drastic changes in the cellular environment generates systemic signals that act in the coordination and execution of responses in terms of metabolic and developmental adjustments. Soluble sugars (namely sucrose, glucose and fructose) that are altered by water deficits and salinity, also act as signalling molecules under stress (**BAJJI et al., 1998; CHAVES et al., 2003**) and interact with PGRs as part of the sugar sensing and signalling network in plants (**JAIN et al., 2007; ROLLAND et al., 2006**). The redox-state of the photosynthetic electron components and the redox-active molecules also act as regulatory agents of stress acclimation metabolism (**FOYER and NOCTOR, 2003**). Adaptation to osmotic and salt stress involves the reprogramming of gene expression, as well as changes in the cell physiology and metabolism. Stress alters the sink-source regulation by switching on genes of sink-specific enzymes in parallel

with stress defence genes upon signal perception (**FEHER et al., 2003**). The resulting metabolic alterations yield a pool of chemically and structurally diverse metabolic compounds as part of the acclimation/adaptive mechanisms.

Primary metabolites linked to amino acids and nitrogen or carbohydrates and polyphenols are reported to increase significantly under moisture and salt stress. These compatible solutes have roles in osmotic adjustment, membrane and protein protection or scavenging of ROS and of excess accumulated ammonium ions (**SPERDOULI and MOUSTAKAS, 2012b**). Osmotic compounds that build up in response to slowly imposed dehydration also have a function in sustaining tissue metabolic activity. Acclimation responses to salinity also include synthesis of compatible solutes as well as adjustments in ion transport (such as uptake, extrusion and sequestration of ions). These responses eventually lead to restoration of cellular homeostasis, detoxification and therefore survival under stress (**CHAVES et al., 2009; SOBHANIAN et al., 2011**). Among primary metabolites produced for plant stress acclimation, proline figures prominently. **HARE and CRESS** (**1997**) and **SHETTY** (**2004**) proposed a model that phenolic metabolites are effectively produced through an alternative mode of metabolism that links proline synthesis with the pentose phosphate pathway (**Figure 1.10**). The proline-linked pentose phosphate pathway stimulates the synthesis of $NADPH_2$, and sugar phosphates for anabolic pathways that include polyphenols and antioxidant response pathways (**HARE and CRESS, 1997**). Proline fulfils diverse functions in plants and its metabolism involves several subcellular compartments and contributes to the redox balance of the cell and mediate osmotic adjustment (**LEHMANN et al., 2010**). However, the cytoplasmic pool of free proline even after the imposition of stress is often insufficient to account for pronounced biophysical effects (**HARE and CRESS, 1997**). Proline accumulation may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy needed for recovery. High levels of proline synthesis during stress may maintain $NAD(P)^+$/NAD(P)H ratios at values compatible with metabolism under normal conditions (**HARE and CRESS, 1997; SHETTY, 2004**). The increased $NADP^+$/NADPH ratio mediated by proline biosynthesis is likely to enhance activity of the oxidative pentose phosphate pathway and thus provide precursors to support the demand for increased secondary metabolite production during stress as well as nucleotide synthesis accompanying the accelerated rate of

cell division upon relief from stress (**HARE and CRESS, 1997; KISHOR et al., 2005**). Oxidation of proline is likely to provide an important energy source for ADP phosphorylation. The reducing equivalents for mitochondrial oxidative phosphorylation are provided by proline, replacing NADH, with oxygen being the terminal electron acceptor. Based on this compelling evidence, can the plant's environment be manipulated to tilt their metabolism in favour of the production of the secondary metabolite of interest? *In vitro* cell culture environments offers an opportunity for such studies. Following this model, proline, its precursors and analogues can be used in *in vitro* cultures to stimulate polyphenolic biosynthesis.

An exponential increase, in polyphenol biosynthesis has been reported under the influence of reduced water availability in a number of plant species (**GLYNN et al., 2004; ALONSO-AMELOT et al., 2007**). A more definite role of phenolic compounds in plant water-relations has been proposed for lipophilic resins accumulated in *Diplacus* and *Larrea* species (**RHOADES, 1977**), where an integrated antidesiccant and UV screen defence role have been assumed. Furthermore, **HORNER** (**1990**) suggested a link between xylem pressure and tannin synthesis, and that the relationship can either be positive or negative, depending on the degree of water stress suffered by the plant.

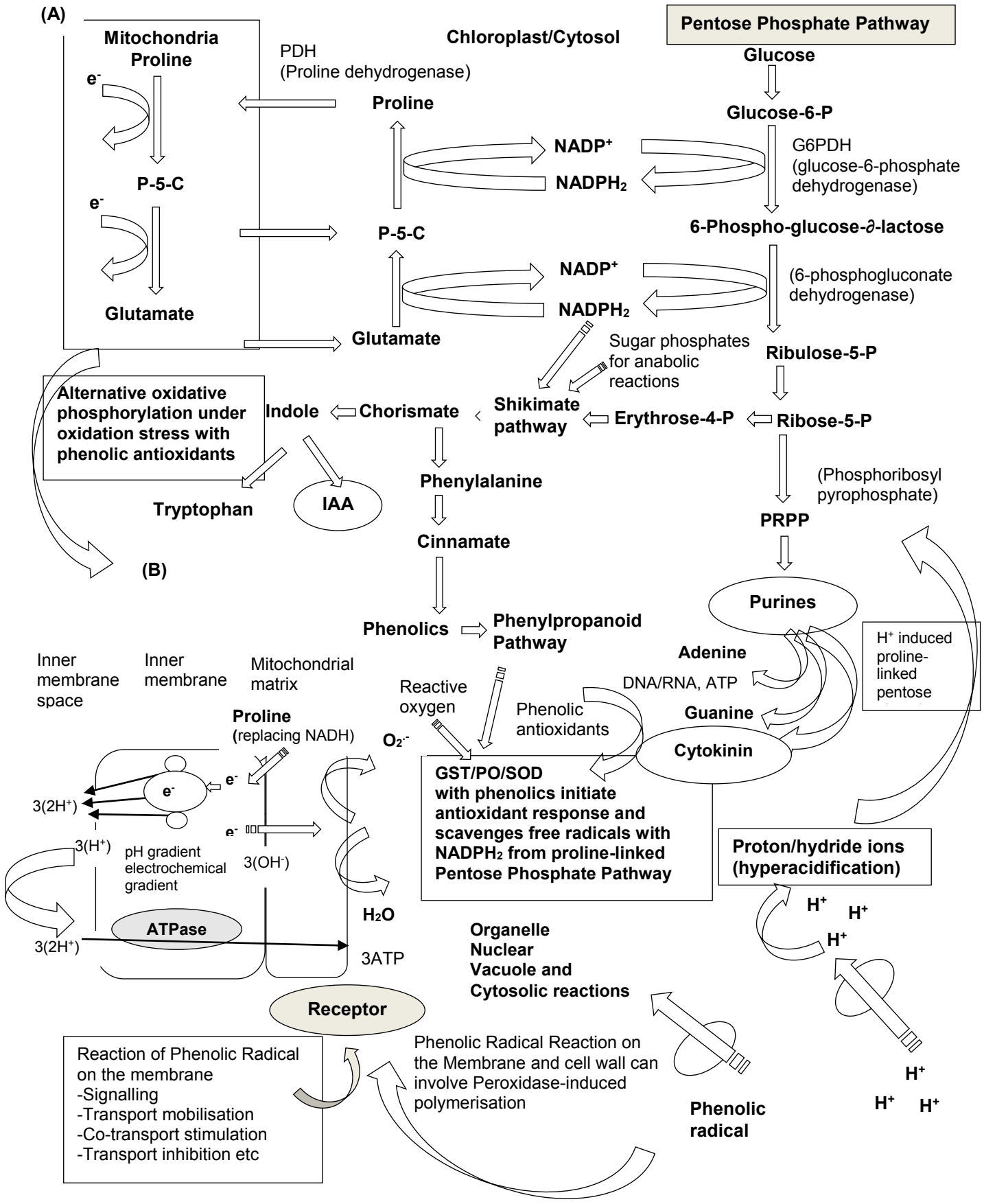


Figure 1.10. Schematic model for the role of proline-linked pentose phosphate pathway in regulating phenolic biosynthesis in plants. The model accommodates the

mechanism of action of external phenolic phytochemicals. P5C, pyrroline-5-carboxylate; IAA, indole acetic acid; GST, glutathione-s-transferase; PO, peroxidase; SOD, superoxide dismutase (**SHETTY, 2004**)

1.7.2. Nutritional stress

The effects of soil nutrient availability on plant growth, physiology, tissue chemistry, or stress tolerance are often investigated by experimentally manipulating nutrient supply. Levels of secondary metabolites in plant tissues are reported to vary with resource availability (**COLEY et al., 1985**). In general, source-sink relations are expected to constrain the levels of metabolites accumulated by growing plants but their dynamics warrants further exploration. Plant nutrient balance in the growth medium is thought to influence the production of secondary compounds at the level of metabolic regulation (**HERMS and MATTSON, 1992**). In particular, nitrogen (N) and carbon (C) serve as structural, molecular and biochemical regulation molecules that dictate growth and assimilate partitioning patterns to various metabolic sinks. Their metabolism in plants are closely interlinked. According to **FRITZ et al**. (**2006**), when the carbon or nitrogen supply is altered, many central metabolites in C and N metabolism change in parallel rather than antagonistically. Nitrogen metabolism involves uptake, transport, assimilation, and utilisation for growth and developmental processes (**LUO et al., 2013**). Each of these processes may be regulated slightly differently, with the resultant differences in N metabolism and plant performance and distinct ecological requirements (**LUO et al., 2013**).

Research demonstrates that nitrate-derived signals are involved in triggering widespread changes in gene expression, resulting in reprogrammed N and C metabolism to facilitate the uptake and assimilation of nitrate, and to initiate accompanying changes in C metabolism (**STITT, 1999**). Nitrate-signalling interacts with signals generated further downstream in N metabolism and C metabolism. In many cases, sugars (C) exert complementary effects to nitrate on gene expression and stimulate the post-translational activation of nitrate reductase (NIA) (**CRAWFORD, 1995; SIVASANKAR et al., 1997**). Low sugar levels exert a strong negative regulation on NIA expression, an effect which over-rides the signals derived from N metabolism (**MORCUENDE et al., 1998; MATT et al., 2002; KOCH, 2004**).

Central metabolism is an important source of precursors for biosynthesis of secondary metabolites. Secondary metabolites have a range of functions in metabolism, signalling and defence against stress factors (**FRITZ et al., 2006**). Nitrogen-rich alkaloids are synthesised from amino acids while C-containing phenylpropanoids are derived from phenylalanine by a reaction sequence that leads to the recycling of ammonium, and terpenoids from acetyl CoA or glycolytic intermediates (**Figure 1.6**). The carbon/nitrogen balance (CNB) hypothesis, as proposed by **BRYANT et al**. (**1983**) postulates that secondary metabolism is directed towards carbon-rich metabolites in nitrogen-limited media, and nitrogen-rich metabolites in carbon-limited regimes. CNB explains the concentrations of secondary metabolites in plant tissues as a function of the relative abundance of plant resources, particularly N. The theory rests on the assumptions that growth (primary metabolism) is a priority for plants over secondary metabolism, and that C and N are allocated to secondary metabolite production only after the requirements for growth are met. It also assumes that the rate of secondary metabolite production is determined by the concentrations of precursor molecules (**REICHARDT et al., 1991**). When growth is limited by N deficiency in plants, CNB predicts that carbohydrates will accumulate in plant tissues, leading to an increased synthesis of carbon-based secondary compounds. The interpretation of the results is that the accumulation of C-based metabolites when the C–N ratio is high is due to a relative excess of fixed carbon being available to the plant. Thus the plant is only able to use the photosynthates for C-, H-, and O-containing metabolites, such as phenolics and terpenes (**WATERMAN and MOLE, 1994**). The theory therefore, predicts that N limitation leads to a decrease in the synthesis of N-containing compounds such as alkaloids and cyanogenic glycosides. Although many studies have yielded results consistent with these predictions (**DIXON and PAIVA, 1995; KOUKI and MANETAS, 2002; MATT et al., 2002; FRITZ et al., 2006; SIMON et al., 2010**), CNB has also failed repeatedly in several other similar studies (**KORICHEVA et al., 1998; RIIPI et al., 2002**).

In line with the opinion of the CNB theory, **JONES and HARTLEY** (**1999**) proposed a protein competition model (PCM) of phenolic allocation in plants. In contrast to the predictions of CNB, the PCM attempts to explain polyphenol biosynthesis on the basis of the metabolic origins of pathway constituents, alternative fates of pathway

precursors, and biochemical regulatory mechanisms. Protein synthesis and the phenylalanine ammonia-lyase (PAL)-catalysed committed step of phenolic and alkaloid biosynthesis, both utilise the amino acid phenylalanine as a precursor (**DIALLINAS and KANELLIS, 1994; KOVÁČIK et al., 2007**). This, therefore, presents a protein-metabolite competition for the limiting phenylalanine, leading to a process-level trade-off between rates of protein versus metabolite synthesis. Due to these demands, the phenylalanine pool appears to be limiting in plants (**WEAVER and HERMANN, 1997**). Allocation to phenolic biosynthesis, therefore, may be determined by the competitive dynamics between protein and phenolic demands as dictated by the inherent growth demands and environmental cues. Because secondary metabolism is linked to primary metabolism by the rates at which substrates are diverted from primary pathways and channelled towards the secondary biosynthetic routes, several factors affecting growth, photosynthesis and other parts of primary metabolism will likely affect levels of secondary metabolites.

Levels of proanthocyanidins were found to increase following limitation in available phosphate (**KOUKI and MANETAS, 2002**) while low iron levels were shown to stimulate increased biosynthesis of a range of polyphenols (**DIXON and PAIVA, 1995**). Furthermore, **MATT et al.** (**2002**) revealed that parallel changes in sugar and amino acid levels correlated with a general increase in the levels of secondary metabolites, irrespective of whether they were carbon-rich or nitrogen-rich. In investigating the effects of soil nutrient factors on salidroside (active ingredient) production in the roots of *Rhodiola sachalinensis* (Chinese medicinal herb), **YAN et al.** (**2004**) found that rich organic matter, low pH and high levels of exchangeable nitrogen and total nitrogen in the soil were essential for high level production of salidroside (a phenolic glycoside). This trade-off in resource allocation between primary and secondary metabolism has important implications on phytochemical accumulation in medicinal plants.

Owing to the multitude of biochemical reactions underlying secondary metabolism and the high degree of connectivity between biochemical pathways and regulatory mechanisms, *in vitro* manipulation of nutrient medium in plant tissue culture offers an enormous potential for mass and novel production of secondary metabolites.

1.8. Significance and aims of the study

The unrestricted collection of medicinal plants from the wild has put many of the slow growing bulbous plant species at the risk of over-exploitation and extinction in South Africa. Conservation of medicinal plant species is important for the sustainability of the plant-based traditional medicine systems as well as the maintenance of biodiversity. This research attempt to ensure a high quality and readily available source of planting material, as a conservation strategy for the species in the wild. This project was aimed at developing micropropagation protocols for the *in vitro* culture and plant regeneration of *Cyrtanthus guthrieae*, *C. obliquus* and *C. contractus* as well as to investigate their physiology and secondary metabolite biosynthesis potential *in vitro*.

The specific objectives of the study were to:

- Determine the optimum chemical and environmental conditions for *in vitro* propagation of each of the three *Cyrtanthus* species;
- Asses the physiological and biochemical responses of the species to environmental stress
- Evaluate the pharmacological properties of the in vitro and ex vitro cultivated plant extracts of the three *Cyrtanthus* species
- Analyse the phytochemical composition of the three species in response to changing environmental regimes

CHAPTER 2

In vitro propagation of *Cyrtanthus* species

2.1. Introduction

SARASAN et al. (**2006**) describes conservation of biodiversity to be most effectively achieved through management of wild populations and their natural habitats. Although the technique appears sound, its practical applicability on a large scale is, however, complicated by the degree of human dependence and interaction with the ecosystem, an aspect that presents a major limitation to its conservation potential. In this human-ecosystem interaction, plants are endangered by a combination of multiple factors which range from over-harvesting, unsustainable agriculture and forestry practices, urbanisation, pollution, habitat destruction, fragmentation and degradation, and climate change (**PAUNESCU, 2009**). As a result, global concern over the loss of valuable genetic resources has stimulated many alternative techniques to complement *in situ* methods. *In vitro* propagation tools and methods are, therefore, among the widely used standard practices in the conservation of genetic diversity of rare and threatened plant species (**REED et al., 2011**).

One striking characteristic of plants is that tissue and organ formation are repetitive processes that occur continuously (**STEEVES and SUSSEX, 1989**). The ability to produce morphologically and developmentally normal whole plants and organs from undifferentiated somatic cells in culture, resides uniquely within the plant kingdom (**ZIMMERMAN, 1993**). It is during embryonic development that the polar axis of the plant is established, domains that set up the organisation of the plant body are defined, and the primary tissue and organ systems are delineated. 'Postembryonic *de novo* organogenesis represents an important strategy that allows physiological and developmental adaptation to changing environmental conditions' (**PERNISOVÁ et al., 2009**). Plant tissue culture techniques, therefore, exploit this inherently unique characteristic of plants to clonally propagate and rapidly multiply plant material. However, this process is influenced and initiated by numerous interacting factors that are able to elicit developmental programs towards the formation of entire organs

from differentiated and undifferentiated cells (**WEST and STOCK, 2001; PERNISOVÁ et al., 2009**).

Manipulation of growth and developmental factors in the culture environment forms the fundamental basis of a successful *in vitro* propagation protocol. Plant growth regulators, particularly auxins and cytokinins (CKs), and their interaction thereof, are among the central factors influencing plant organogenesis (**SKOOG and MILLER, 1957**). In their pioneering work, **SKOOG and MILLER** (**1957**) identified auxin-to-CK concentration ratios as an important factor regulating the developmental fate of plant tissue explants. Since then, many of the investigations aimed at optimising micropropagation protocols have centred on the choice and fine-tuned combination of these phytohormones in culture (**WERBROUCK et al., 1995; McALISTER et al., 1998; TANAKA et al., 2006**). In addition, the development of the MS medium (**MURASHIGE and SKOOG, 1962**) provided some further insights into the many interactions of minerals in the medium and their uptake by plant tissues, with the resulting responses measured at each interval stage. Optimal responses provide practical information that can be determined from different types of formulations. In principle, however, the generally complex phenomenon of organogenic response of plant explants in culture is under the influence of PGR, environmental and nutritional factors and explant genotype among other factors (**TSUKAYA and BEEMSTER, 2006**). Components of tissue culture media at different concentrations exert their effects on growth and morphogenesis of the cultured plant cells, tissues and organs due partially to their nutritional value and to altering its osmotic potentials (**DE PAIVA NETO and OTONI, 2003**). **LAPEÑA et al.** (**1988**), for example, estimated that three quarters of the sucrose necessary to promote an optimal rate of adventitious shoot formation in *Digitalis obscura* hypocotyls were required for energy supply, while the excess osmotically regulated morphogenesis.

A number of micropropagation protocols have been optimised for different wild and cultivated plant species. According to **REED et al.** (**2011**), species that are at risk of extinction due to rapid loss of genetic diversity and habitat come mainly from resource-poor areas of the world and from global biodiversity hotspots. *Cyrtanthus* species are among such a group of plants in southern Africa, with only a few small populations remaining in the wild for some species. This study was aimed at

developing effective micropropagation protocols for *C. contractus, C. guthrieae* and *C. obliquus* as a step towards their conservation. The effects of different PGRs, media components and photoperiods were investigated in this study.

2.2. Materials and methods

2.2.1. Explant decontamination and shoot induction

The primary explants used were twin scales excised from mature *Cyrtanthus* stock plants maintained in the shade house at the University of KwaZulu-Natal (UKZN) Botanical Gardens, Pietermaritzburg, South Africa. The bulbs were thoroughly washed under running tap water to remove soil and the brown outermost scales peeled away to expose the fresh inner scales. Peeled and trimmed bulbs were surface decontaminated using 70% ethanol (v/v) for either 1 or 2 min followed by 1% (w/v) Benlate® for either 10 or 15 min, 0.2% (w/v) mercuric chloride ($HgCl_2$) solution for either 10 or 15 min and rinsed three times with sterile distilled water. Each solution was supplemented with a few drops of Tween 20® to act as a surfactant. Bulbs were then cut in half and soaked in 0.2% $HgCl_2$ for 10 min after which they were washed with three changes of sterile distilled water. Twin-scales joined by 5 mm of the basal plate were excised from these cut bulb segments and cultured individually adaxial side down onto 10 ml sterilised full strength **MURASHIGE and SKOOG** (**1962**) (MS) medium in culture tubes (100 mm x 25 mm, 40 ml volume). The MS medium was supplemented with 0.1 g l^{-1} myo-inositol, 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar (Bacteriological agar-Oxoid Ltd., Basingstoke, Hampshire, England). A 4 x 5 factorial experiment with different combinations and concentrations of α-naphthalene acetic acid (NAA) (0, 0.5, 1.1, 2.7 µM) and 6-benzyladenine (BA) (0, 1.1, 4.4, 6.7, 8.9 µM) was conducted. The pH of the medium was adjusted to 5.8 with either KOH or HCl prior to autoclaving at 121 °C and 103 kPa for 20 min. Cultures were maintained in a growth room with cool white fluorescent tubes (Osram® L 58 W/640, Germany) at 16 h photoperiod, with a PPFD of 60 µmol m^{-2} sec^{-1} at 25 ± 2 ºC. A similar set of treatments was maintained under similar conditions of temperature but with continuous darkness. Each treatment was repeated twice with each replicate consisting of 24 twin-scale explants. After eight weeks of culture, the number of shoots produced per explant, decontamination

frequency (%) in each treatment and shoot lengths (cm), were recorded. Decontamination of bulb scales was also attempted using 3.5 % (v/v) sodium hypochlorite but however, this treatment procedure was unsuccessful.

2.2.2. Effects of types and concentrations of cytokinins on shoot proliferation

Five CKs, BA, *meta*-topolin (mT), kinetin (Kin), thidiazuron (TDZ) and zeatin (ZT) were used to evaluate shoot multiplication and *in vitro* plant growth at three concentrations (1.0, 5.0 and 10.0 µM) in a complete randomised design (CRD). A set of cultures without any PGR supplement served as a control for each *Cyrtanthus* species. In addition, the media that gave the highest number of shoots for each species in the shoot initiation experiments were also included as part of the treatments. BA, Kin, TDZ and ZT were purchased from Sigma-Aldrich (USA) while mT was obtained from the Laboratory of Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, Czech Republic. Shoots obtained from the previous experiment (**Section 2.2.1**) were used as explants and maintained in a growth room with 16 h photoperiod and 60 µmol m^{-2} sec^{-1} PPFD at 25 ± 2 ºC. Each treatment was repeated twice with 24 explants per replicate. Total number of adventitious shoots produced per explant, shoot fresh and dry mass, as well as shoot length was recorded after 8 weeks of culture. The combined results from the two separate experiments were used for data analysis.

2.2.3. Effects of photoperiod on shoot multiplication

Based on the results obtained from the preceding experiment (**Section 2.2.2**), shoot explants excised from the media with the highest shoot proliferation rates, for each species were cultured on the same medium. The cultures were divided into two, each with 40 replications and maintained under continuous light and 16 h light/dark conditions respectively at 25 °C (60 µmol m^{-2} sec^{-1} PPFD in each case). Shoot multiplication frequency, shoot fresh weight and dry mass, together with shoot length were recorded after 8 weeks of culture.

2.2.4. Effects of sucrose concentration on bulblet growth and multiplication

To investigate the effects of sucrose concentration on bulblet biomass accumulation, shoot clusters produced in the optimum shoot multiplication medium (**Section 2.2.2**) were carefully separated and used for this experiment. The shoot explants were transferred into screw cap culture jars (110 mm × 60 mm, approximately 300 ml volume) containing 30 ml of MS medium supplemented with 10, 30, 60, 90 and 120 g l^{-1} sucrose concentrations. To avoid vitrification problems, explants were recultured every four weeks and biomass accumulation, shoot multiplication and bulblet sizes were measured after 12 weeks of culture. Each treatment had 24 explants and the culture conditions were as described in **Section 2.2.2.**

2.2.5. Shoot multiplication via indirect organogenesis

2.2.5.1. Callus induction

An experiment on precursor feeding in an investigation of *in vitro* secondary metabolites (**Section 3.3.2.1**) generated substantial amounts of callus for *C. guthrieae.* Concentrations of 10, 50, 100 and 1000 µM of alkaloid and polyphenol biosynthetic intermediate precursors (phenylalanine and tyrosine) were added as supplements to the optimum shoot multiplication MS media. Of these treatments, MS medium supplemented with 100 µM phenylalanine induced friable callus production on *C. guthrieae* shoot explants (**Figure 2.4**) maintained under conditions similar to those described in **Section 2.2.2***.*

2.2.5.2. Indirect shoot organogenesis

The organogenic calli was subcultured for proliferation on the same medium (described in **Section 2.2.5.1**) for 4 weeks. After sufficient bulking, callus pieces of known quantity were transferred to the shoot regeneration MS medium (**Section 2.2.1**) for *C. guthrieae* supplemented with 4.4 µM BA and 1.1 µM NAA. The morphogenic effects of various PGR combinations on calli were further evaluated in a complete randomised design with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), picloram and BA (**Table 2.4**) in a factorial

treatment structure. Uniform pieces of calli (100 mg) were cultured in Erlenmeyer flasks (250 ml) containing 40 ml liquid MS medium each with different PGR treatments and placed on a rotary shaker at 100 rpm. Each treatment was replicated twice with 8 flasks in each replicate. The number of adventitious microshoots produced per callus (≥ 0.5 mm) and shoot regeneration frequency (%) in each treatment were recorded after 8 weeks. The callus-regenerated shoots were transferred to the optimised solid shoot regeneration MS medium (as described in **section 2.2.1**) for *C. guthrieae* for multiplication. The culture environmental conditions were similar to those described in **Section 2.2.2.**

2.2.6. *In vitro* rooting of regenerated shoots

In vitro regenerated shoots from the optimised shoot multiplication media were evaluated for rooting. Individual shoots were transferred to half- and full-strength MS medium without any PGR supplement. The cultures were completely randomised and maintained in a growth room with culture conditions as described in **section 2.2.2.** Each treatment was repeated twice with each replicate consisting of 24 individually cultured shoot explants. The data from the two replicates were combined for data analysis. Data on root induction frequency (%), number of roots produced per shoot explant and root length were recorded after 4 weeks in culture.

2.2.7. *Ex vitro* acclimatisation of rooted plants

In vitro rooted plants were potted in plastic containers in a mixture of sand-soil-vermiculite (1:1:1, v/v/v), and maintained in a mist house with high relative humidity (90-100%) for three days. The midday PPFD in the misthouse ranged between 90 and 100 µmol m^{-2} s^{-1} with natural photoperiod conditions. The plants were then transferred to the greenhouse set at 25 ºC and a midday PPFD of approximately 1 000 µmol m^{-2} s^{-1}. The percentage of surviving plants was recorded after two weeks in the greenhouse.

2.2.8. Statistical analysis

All data were subjected, as appropriate, to either one-way analysis of variance (ANOVA) or *t*-test using either SPSS software for Windows (IBM SPSS®, version 21.0, Chicago, USA) and/or GenStat 14th edition (VSN International, UK). Percentage data were arcsine transformed prior to analysis with respective statistical tests. Significantly different means ($P \leq 0.05$) were further separated using Duncan's multiple range test (DMRT) and/or Least Significant Difference (LSD).

2.3. Results and discussion

2.3.1. Explant decontamination

The decontamination frequency recorded for the bulb explants of the three *Cyrtanthus* species is shown in **Figure 2.1.** the highest decontamination frequency, in all three species, was obtained from the treatment involving soaking in 70% ethanol for 2 min followed by 1% Benlate for 15 min and 0.2% $HgCl_2$ for 15 min before being cut into half and soaked further in 0.2% $HgCl_2$ for 10 min. This decontamination procedure gave frequencies of 78, 93 and 88% for *C. contractus*, *C. guthrieae* and *C. obliquus* respectively. An increase in the duration of soaking in 0.2% $HgCl_2$ solution from 10 to 15 min resulted in a significant increase in the decontamination frequency for *C. contractus* although only a slight but insignificant increase were recorded on *C. guthrieae* and *C. obliquus* explants. The use of geophyte storage organs as culture explants is often associated with pathogenic contamination problems (**MACDONALD, 1987**), thus the use of harsh sterilants as mercuric chloride for aseptic treatments in this study.

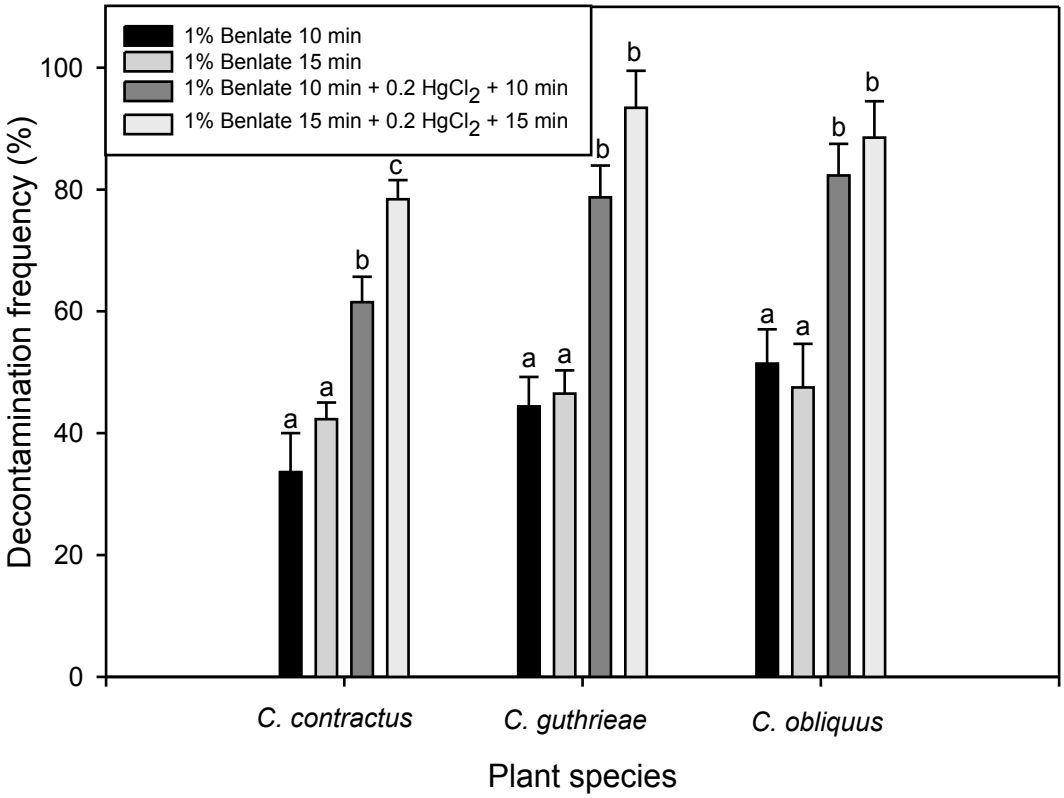


Figure 2.1. Effects of different treatment procedures on the decontamination frequency of three *Cyrtanthus* twin-scale explants. Each treatment procedure was preceded by soaking in 70% ethanol

2.3.2. Effects of BA and NAA on shoot multiplication

The number of shoots initiated per explant in each of the three species varied significantly among the different auxin and cytokinin combinations (**Figure 2.2**). Although shoot induction occurred in almost all the treatments in this study, twin scale explants cultured on MS medium supplemented with a PGR combination of 4.4 µM BA and 1.1 µM NAA produced the highest number of shoots/explant (2.6 and 6.3) for *C. contractus* and *C. guthrieae* respectively, each with a regeneration frequency of above 80%. On the other hand, in *C. obliquus*, the highest shoot induction rate (1.9 shoots/explant) was recorded in slightly higher CK and auxin concentration levels (6.7 µM BA and 2.7 µM NAA respectively). The low concentration level of PGR requirements for shoot induction in *C. contractus* and *C. guthrieae* may suggest that the two species contain higher endogenous levels of PGR to induce shoot formation compared to *C. obliquus* (**MAESATO et al., 1994**).

The interactive effect of an exogenous supplement of auxins and CK on shoot morphogenesis in cultured explants is exerted through the alteration of their endogenous balance within the explant (**BAJGUZ and PIOTROWSKA, 2009**). An optimally adjusted balance between the two PGRs shifts morphogenic development of cultured cells significantly towards shoot development. In the absence of NAA, an increase in BA concentration resulted in an insignificant increase in adventitious shoot induction compared to a corresponding increase in combination with NAA. One possible explanation to this phenomenon could be that the increased exogenous NAA may be up-regulating CK levels by promoting BA metabolic activation through *N*-glucosylation (**AVALBAEV et al., 2012**) and hence shoot organogenesis. The highest number of shoots as well as the percentage of explants producing shoots in *C. loddigesianus* and *C. speciosus* was achieved with high doses of NAA/BA (5.37 µM/8.8 µM) (**ANGULO et al., 2003**). The results are consistent with those observed by other reseachers on other *Cyrtanthus* species (**McALISTER et al., 1998; MORÁN et al., 2003; HONG and LEE, 2012**), other members of the Amaryllidaceae (**SLABBERT et al., 1993; VISHNEVETSKY et al., 2003**) and for bulbous species from other families (**CHEESMAN et al., 2010**). While a number of research findings are consistent with this observed trend on bulb explants, several others have yielded results in sharp contrast. For example, **SLABBERT and NIEDERWIESER** (**1999**), **FENNELL** (**2002**) and **RICE et al.** (**2011**) reported the highest shoot and bulblet induction respectively from twin scales cultured on MS medium free of exogenous PGR, an assertion that further confirms the significant role of the endogenous content of phytohormones and their interplay with exogenous supply in regulating morphogenic parameters in plant cells.

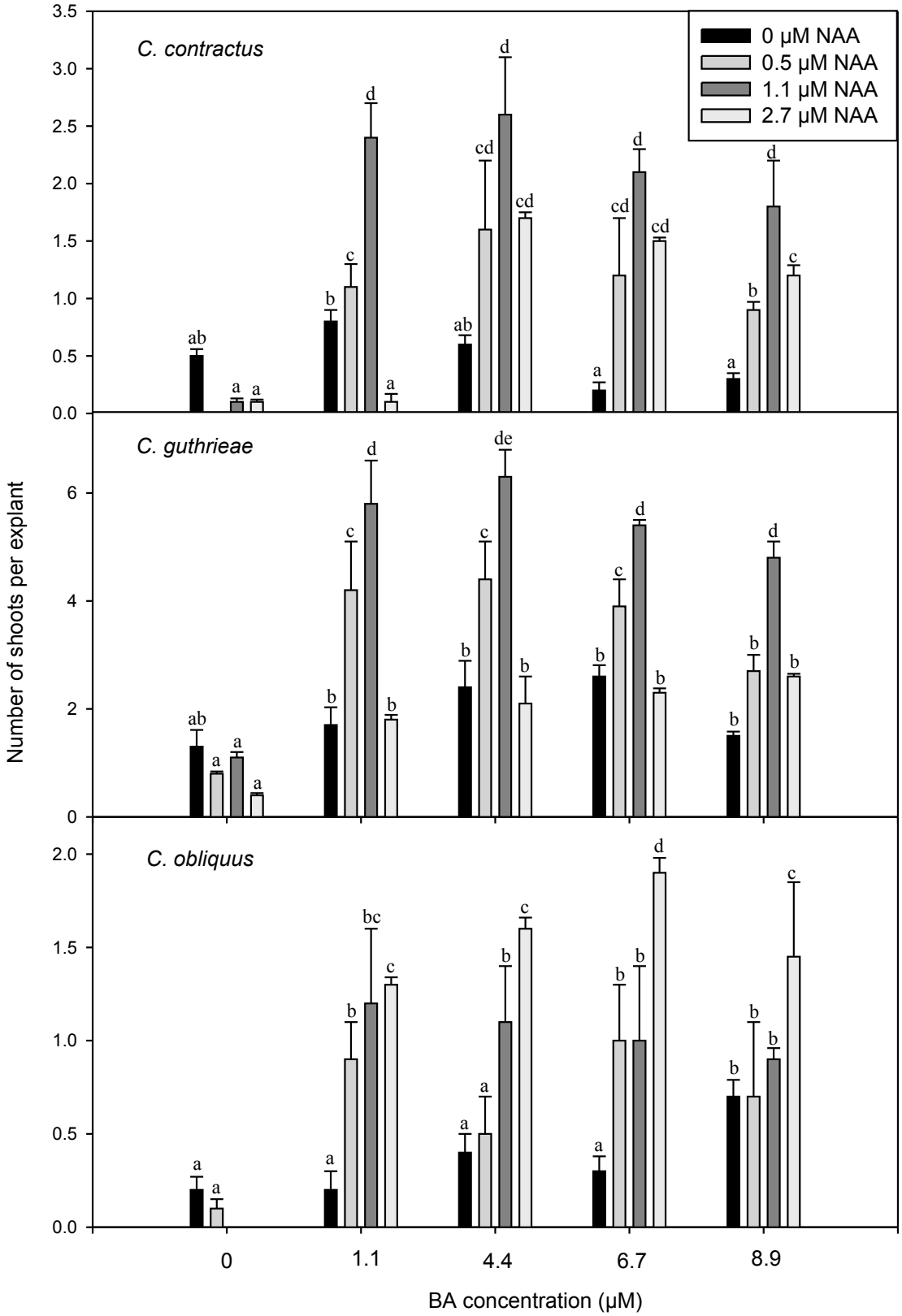


Figure 2.2. Effects of BA and NAA interaction on shoot regeneration of *Cyrtanthus contractus, C. guthrieae* and *C. obliquus* after eight weeks of culture. Bars with different letters are significantly different ($P \leq 0.05$) according to DMRT

Although most shoots in all three species were derived from the basal plates, some explants, particularly those of *C. contractus* and *C. guthrieae*, however, produced shoots directly from twin-scales (**Figure 2.3A**). Anatomical observations on bud regeneration in *Nerine bowdenii* revealed that buds form on twin scale explants that contain basal-plate tissue as a result of outgrowth of pre-existing axillary meristems and the regeneration of adventitious buds in the axils of the scales (**GROOTAARTS et al., 1981; VISHNEVETSKY et al., 2003**). A similar mechanism of shoot regeneration as that described for *N. bowdenii* could be assumed to have been responsible for the characteristic shoot induction observed in this study. The number of shoots produced from axillary buds is however, limited to the number of pre-existing meristems placed in each culture while a greater potential for multiplication exist with adventitious buds as shoots may arise from any part of the explant (**ZIMMERMAN, 1993**), an attribute that offers promising potential for further manipulation of *Cyrtanthus* scale explants for improved shoot regeneration. In the shoot induction experiment under continuous darkness treatment in this study, the only responsive explants were from *C. contractus* but with significantly lower regeneration rates compared to those cultured in a 16 h photoperiod. In addition, only achlorophyllous axillary shoots from the basal plates were recorded, with no adventitious shoots from the scales, further indicating the influence of light in this morphogenic development process. The deductions drawn from the experimental results of this study is that the uptake of BA and NAA from the medium by the twin scale explants of the three *Cyrtanthus* species, represents an essential requirement the shoot organogenesis process.

2.3.3. Effects of types and concentrations of cytokinins on shoot proliferation

Different types and concentrations of CK treatments differentially influenced shoot proliferation in three *Cyrtanthus* species (**Table 2.1**). With the exception of 1.0 µM Kin on *C. contractus* and all the ZT on *C. guthrieae*, shoot multiplication increased at all CK concentrations compared to the PGR free controls in all three species. As opposed to the other two species, an increase in BA concentration from 1.0 to 10.0 µM led to a decline in the number of shoots produced per explant in *C. guthrieae*. The highest number of shoots/explant from the media supplemented with different types and concentrations of CKs were recorded at 10.0 µM TDZ for *C. contractus*

(7.5), 5.0 µM TDZ for *C. guthrieae* (15.0) (**Figure 2.3E**) and 10.0 µM BA for *C. obliquus* (4.0). Thidiazuron, a substituted phenyl-urea, has proven to be a highly effective PGR in the various morphogenetic and embryogenic responses of different plant species (**MITHILA et al., 2001; JONES et al., 2007; ARSHAD et al., 2012; BASKARAN et al., 2012**). Characteristically, TDZ is a non-expensive and potent CK, the high activity of which may be attributed partly to its extreme stability in plant tissues (**MOK et al., 2000**). Its mode of action may be effected either direct or indirect, by regulating endogenous CK biosynthesis and/or metabolism (**MOK et al., 2000**). In sugar beet, TDZ-induced somatic embryogenesis occurred on the lower surface of cotyledons at a concentration of 0.5 mM and was found to be less genotype-dependent than with BA (**ZHANG et al., 2001**). The superior effects of TDZ over other CKs on shoot proliferation of *C. contractus* and *C. guthrieae* is consistent with the previous findings from other plant species and suggest that this CK may substitute for BA in the tissue culture of these plant species.

A mixture of auxin/CK (1.1 µM NAA/4.4 µM BA) however, proved to be an excellent PGR media supplement for shoot proliferation compared to any of the tested CKs applied singularly on *C. guthrieae* (**Table 2.1**). The combination resulted in the highest number of shoot frequency for this species although not significantly different from the 5.0 µM TDZ treatment. The synergistic interaction effect of auxins with CKs on shoot multiplication has similarly been reported for other *Cyrtanthus* species (**ANGULO et al., 2003; MORÁN et al., 2003**). However, none of these previous researchers investigated the effect of different CKs on shoot morphogenesis of *Cyrtanthus* species. The role of PGRs on the physiomorphogenic developmental patterns of cultured cells has always been inferred mostly from the effects of exogenous applications, often with poor understanding of their mechanisms of action. **NORDSTRÖM et al.** (**2004**), however, argues that auxin controls CK biosynthesis via the specific activation of *IPT5* and *IPT7* genes (**MIYAWAKI et al., 2004**) and that most auxin-resistant mutants also show changes in their CK sensitivity (**NORDSTRÖM et al., 2004; IOIO et al., 2008**). On the other hand, **PERNISOVÁ et al.** (**2009**) showed that CK modulates auxin-induced organogenesis via regulation of the efflux-dependent intercellular auxin distribution. Using a hypocotyl explants-based *in vitro* system to study the mechanism underlying *de novo* organogenesis, the authors demonstrated that auxin, but not CK, is capable of

triggering organogenesis in hypocotyl explants. The auxin-induced organogenesis is accompanied by endogenous CK production and tissue-specific activation of CK signalling. Cytokinin affects differential auxin distribution, and the CK-mediated modulation of organogenesis is simulated by inhibition of polar auxin transport (**PERNISOVÁ et al., 2009**). This interdependent hormonal network gets integrated at multiple levels, such as during auxin signalling, metabolism, and carrier-dependent distribution, with the resulting positive morphogenic developments. Following this logic, the same mechanism of action and the integrated effects could thus be assumed in the excellent shoot proliferation demonstrated by a treatment combination of NAA and BA in *C. guthrieae* compared to different CKs alone. Based on these findings, one of the logical deductions to be drawn is that auxin acts as an intrinsic trigger of the organogenic processes, whose output is modulated by the endogenous levels of CK.

Table 2.1. Effect of plant growth regulators on *in vitro* adventitious shoot proliferation of *Cyrtanthus* species after 8 weeks in culture

Plant species	Cytokinin	(µM)	No. of shoot/explant	Fresh mass (mg)	Dry mass (mg)	Shoot length (cm)
C. contractus	BA	1.0	3.4 ± 0.13^{cd}	151.1 ± 4.75^{fg}	13.8 ± 0.69^{ef}	7.9 ± 0.66^{bc}
		5.0	5.7 ± 0.25^{f}	160.4 ± 2.70^{g}	14.5 ± 0.73^{f}	5.7 ± 0.53^{ab}
		10.0	5.9 ± 0.21fg	125.0 ± 3.85^{de}	11.0 ± 0.85^{d}	5.0 ± 0.34^{a}
	*m*T	1.0	2.0 ± 0.16^{ab}	275.8 ± 5.95^{i}	19.4 ± 1.45^{h}	11.0 ± 0.60^{d}
		5.0	2.4 ± 0.13^{b}	192.9 ± 7.76^{h}	17.8 ± 1.28^{gh}	6.8 ± 0.76^{abc}
		10.0	3.5 ± 0.20^{cde}	97.8 ± 5.39^{b}	7.6 ± 0.87^{abc}	7.0 ± 0.51^{abc}
	Kin	1.0	1.5 ± 0.13^{a}	130.2 ± 3.04^{e}	11.1 ± 1.15^{d}	7.9 ± 0.49^{bc}
		5.0	3.1 ± 0.11^{c}	124.5 ± 2.88^{de}	9.6 ± 1.06^{bcd}	6.6 ± 0.33^{abc}
		10.0	3.4 ± 0.13^{cd}	94.4 ± 3.64^{b}	7.5 ± 0.74^{ab}	6.1 ± 0.39^{abc}
	TDZ	1.0	3.1 ± 0.17^{c}	114.1 ± 5.08^{cd}	10.3 ± 0.95^{cd}	7.1 ± 1.23^{abc}
		5.0	3.8 ± 0.13^{de}	104.8 ± 7.85^{bc}	10.0 ± 0.30^{bcd}	5.4 ± 0.58^{a}
		10.0	7.5 ± 0.30^{h}	74.5 ± 5.30^{a}	6.3 ± 0.43^{a}	6.4 ± 0.93^{abc}
	ZT	1.0	3.7 ± 0.16^{de}	162.0 ± 3.22^{g}	16.0 ± 0.58^{fg}	5.8 ± 0.73^{ab}
		5.0	4.0 ± 0.14^{e}	144.5 ± 3.44^{f}	15.2 ± 0.57^{g}	4.8 ± 0.54^{a}
		10.0	6.3 ± 0.15^{g}	180.1 ± 3.48^{h}	11.4 ± 0.81^{de}	8.2 ± 1.12^{c}
	4.4 µM (BA) 1.1 µM (NAA)		6.8 ± 0.21^{g}	183.8 ± 4.62^{h}	9.8 ± 1.09^{bcd}	6.2 ± 0.52^{abc}
	Control		1.9 ± 0.15^{ab}	185.7 ± 3.36^{h}	18.0 ± 0.88^{gh}	12.7 ± 0.74^{d}
C. guthrieae	BA	1.0	10.4 ± 0.29^{g}	121.1 ± 2.73^{bc}	10.6 ± 1.01^{cd}	10.1 ± 0.55^{de}
		5.0	9.0 ± 0.13^{f}	123.3 ± 2.50^{c}	7.9 ± 0.61^{ab}	8.9 ± 0.50^{cde}
		10.0	6.5 ± 0.17^{d}	94.5 ± 3.93^{a}	6.8 ± 0.23^{a}	6.6 ± 0.48^{abc}
	*m*T	1.0	6.9 ± 0.18^{de}	141.2 ± 3.49^{fg}	10.8 ± 0.48^{cde}	10.6 ± 0.84^{e}
		5.0	7.0 ± .018de	136.1 ± 3.06^{def}	12.9 ± 0.59^{efg}	9.0 ± 0.65^{cde}
		10.0	7.4 ± 0.20^{e}	112.3 ± 4.94^{b}	11.4 ± 0.67^{cde}	7.7 ± 0.65^{abcd}
	Kin	1.0	3.3 ± 0.23^{c}	149.5 ± 3.43^{hij}	14.2 ± 1.10^{gh}	9.2 ± 0.95^{de}
		5.0	3.5 ± 0.15^{c}	146.7 ± 2.64^{g}	13.6 ± 0.61^{fgh}	7.8 ± 0.76^{bcd}
		10.0	3.7 ± 0.22^{c}	123.5 ± 3.73^{c}	11.2 ± 0.56^{cde}	9.4 ± 0.92^{de}
	TDZ	1.0	14.0 ± 0.16^{h}	157.6 ± 2.43^{hi}	9.5 ± 0.55^{bc}	8.3 ± 1.02^{bcde}
		5.0	15.0 ± 0.16^{i}	126.4 ± 3.61^{cd}	8.1 ± 1.12^{ab}	8.0 ± 1.33^{bcde}
		10.0	10.0 ± 0.20^{g}	158.8 ± 2.63^{i}	11.1 ± 0.50^{cde}	5.4 ± 0.48^{a}
	ZT	1.0	2.2 ± 0.14^{ab}	148.0 ± 2.77^{gh}	11.8 ± 0.58^{def}	9.1 ± 0.71^{cde}
		5.0	1.7 ± 0.18^{a}	131.0 ± 3.23^{cde}	12.0 ± 0.72^{def}	7.9 ± 0.67^{bcd}

Plant species	Cytokinin	(µM)	No. of shoot/explant	Fresh mass (mg)	Dry mass (mg)	Shoot length (cm)
		10.0	2.0 ± 0.14^{ab}	139.7 ± 2.89^{efg}	13.0 ± 0.64^{efg}	6.1 ± 0.39^{ab}
	4.4 µM (BA) 1.1 µM (NAA)		15.8 ± 0.14^{i}	123.4 ± 2.53^{c}	10.0 ± 0.68^{bc}	5.8 ± 0.42^{ab}
	Control		2.3 ± 0.22^{b}	193.1 ± 3.60^{j}	15.5 ± 0.45^{h}	10.2 ± 0.96^{de}
C. obliquus	BA	1.0	2.1 ± 0.26^{abcd}	125.4 ± 3.41^{efg}	8.6 ± 0.59^{ef}	5.7 ± 0.58^{ab}
		5.0	3.1 ± 0.23^{ef}	120.3 ± 3.44^{efg}	9.0 ± 0.45^{defg}	5.2 ± 0.80^{a}
		10.0	4.0 ± 0.24^{g}	87.1 ± 3.43^{b}	6.7 ± 0.22^{ab}	4.8 ± 0.64^{a}
	*m*T	1.0	2.5 ± 0.26^{cde}	93.3 ± 3.48^{bc}	6.9 ± 0.23^{abc}	6.8 ± 0.73^{ab}
		5.0	2.5 ± 0.28^{cde}	75.0 ± 2.01^{a}	5.8 ± 0.25^{a}	6.5 ± 0.44^{ab}
		10.0	2.6 ± 0.19^{cde}	84.7 ± 3.01^{b}	7.0 ± 0.52^{abc}	7.0 ± 0.76^{abc}
	Kin	1.0	1.9 ± 0.18^{abc}	126.6 ± 4.17^{efg}	10.1 ± 0.38^{fgh}	9.1 ± 0.50^{c}
		5.0	1.9 ± 0.18^{abc}	118.3 ± 2.68^{de}	9.7 ± 0.30^{efgh}	7.8 ± 1.01^{bc}
		10.0	2.4 ± 0.23^{bcd}	92.7 ± 2.95^{bc}	7.8 ± 0.19^{bcd}	6.3 ± 0.65^{ab}
	TDZ	1.0	3.4 ± 0.20^{fg}	129.8 ± 4.16^{fg}	9.5 ± 0.63^{efg}	5.4 ± 0.87^{ab}
		5.0	3.5 ± 0.28^{fg}	134.1 ± 1.87^{gh}	10.4 ± 0.70^{gh}	5.3 ± 1.02^{a}
		10.0	3.6 ± 0.26^{fg}	140.4 ± 4.76^{hi}	9.4 ± 0.92^{efg}	5.3 ± 0.43^{a}
	ZT	1.0	1.6 ± 0.19^{a}	99.2 ± 2.29^{c}	8.7 ± 0.18^{def}	6.4 ± 0.78^{ab}
		5.0	2.6 ± 0.23^{cde}	100.7 ± 4.22^{c}	7.8 ± 0.29^{bcd}	6.1 ± 0.36^{ab}
	6.7 µM (BA) 2.7 µM (NAA)		3.6 ± 0.33^{fg}	133.4 ± 3.61^{gh}	9.4 ± 1.11^{efg}	5.3 ± 0.93^{a}
		10.0	2.7 ± 0.18^{de}	114.1 ± 1.95^{d}	8.3 ± 0.40^{cde}	5.4 ± 0.96^{a}
	Control		1.6 ± 0.17^{ab}	146.9 ± 2.81^{i}	11.0 ± 0.35^{h}	9.1 ± 0.60^{c}

Mean values ± standard error (n = 24) in the same column for each species with different letter(s) indicate a significant difference ($P \leq 0.05$) based on DMRT

Despite several reports emphasising the superior ability of *m*T on shoot proliferation, *in vitro* morphogenesis and/or plantlet quality over other aromatic CKs in a number of plant species (**BOGAERT et al., 2004; BAIRU et al., 2007**; **MOYO et al., 2012**), its effects on the three *Cyrtanthus* species in this study were comparatively lower by a significant magnitude. On the other hand, related studies report kinetin, even at high concentrations, to yield comparatively low adventitious shoot regeneration (**BECK and CAPONETTI, 1983**), results which agree with the findings of this study. However, visually superior (in terms of plant texture, stem thickness, vigour) quality shoots were obtained from treatments with *m*T and Kin (**Figure 2.3C & D**) in all three species although their effect on shoot multiplication was significantly lower than

those for BA and TDZ. In addition to producing good quality plantlets, Kin also exhibited some auxin-like activity by inducing rooting and callus on *C. contractus* and *C. guthrieae* plantlets (**Figure 2.3B**). Formation of lateral roots represents one of the examples of postembryonic *de novo* organogenesis in plants. Recent studies (**LAPLAZE et al., 2007; PÉRET et al., 2009**) suggest potential involvement of CKs in the regulation of lateral root formation, a phenomenon which could have facilitated this process in the two species with Kin treatment in this study. The shoots produced from TDZ treatments were of equally good quality although not as good as those obtained from *m*T and Kin. However, considering the inexpensive nature of TDZ, its stability in plant tissues and the number and quality of shoots induced in *C. guthrieae*, the choice for its use ahead of the other tested CKs for this plant species would thus be a logical option.

Although there were no distinct trends observed on shoot biomass and shoot length, in relation to the type and concentration of CKs, there seemed to be a general trade-off between shoot proliferation rate and other growth parameters in all three species. Control treatments, most of which had lower shoot multiplication frequency, recorded the highest shoot biomass and shoot length. In *C. guthrieae*, the medium containing 5.0 µM TDZ, despite inducing a 7-fold higher number of shoots (15) than the control (2.3), their lengths did not however, differ significantly (**Table 2.1**). The high CK activity and positive response of these and other species (mostly woody species) to TDZ have established it as among the most important CKs for *in vitro* manipulation of plant species.

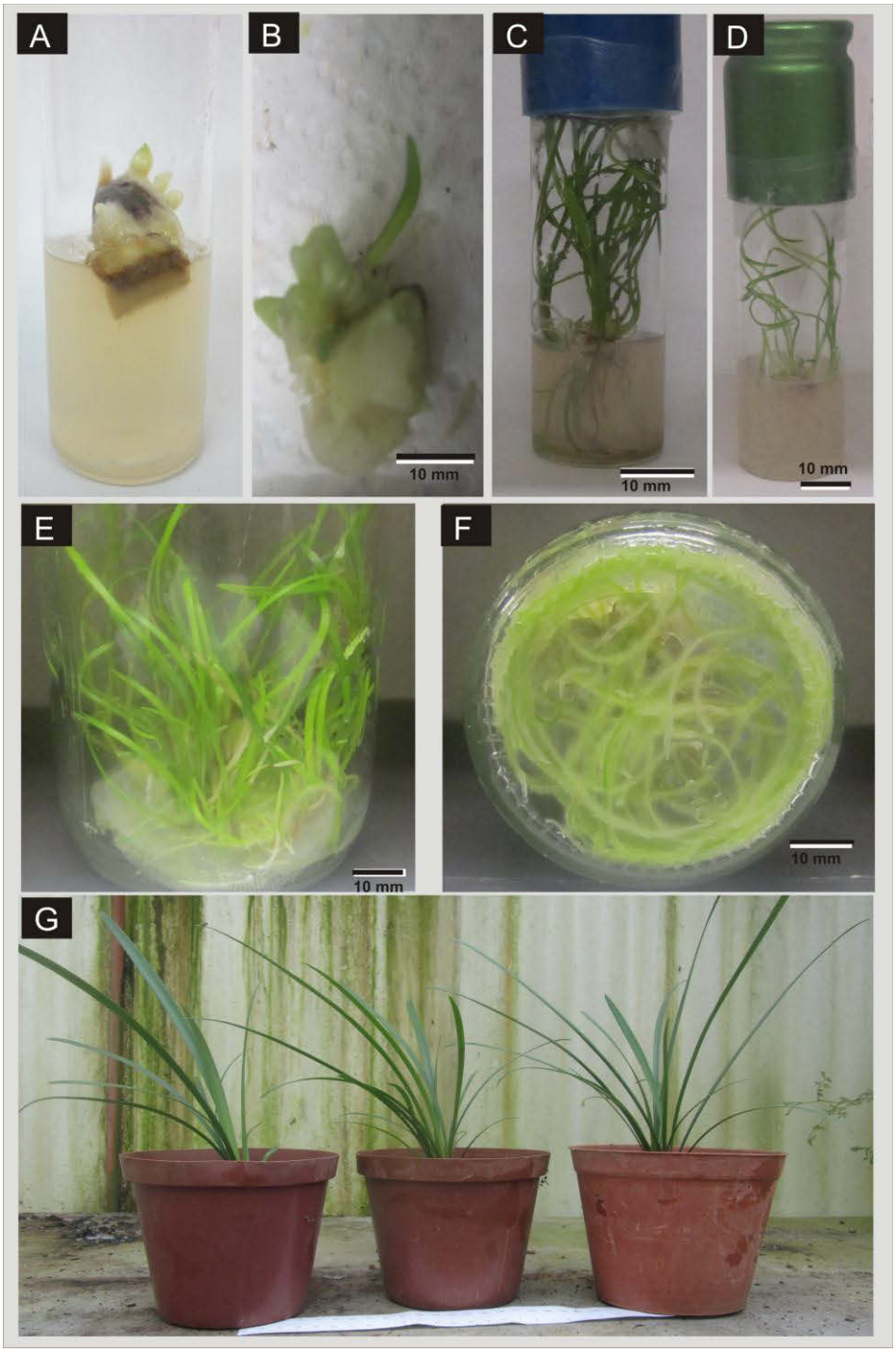


Figure 2.3. *In vitro* culture and *ex vitro* acclimatisation of *Cyrtanthus* species. (**A**) *In vitro* shoot development on the bulb scales of *C. contractus* after 4 weeks in culture. (**B**) Shoot organogenesis from *C. contractus* callus derived from media supplemented with 10 µM Kin. (**C**) Visually superior quality *C. obliquus* plantlets derived from 5 µM mT MS medium supplement after 8 weeks in culture. (**D**) Thin and pale *C. obliquus* plantlets derived from 1 µM ZT MS medium supplement after 8 weeks. (**E**) Shoot multiplication of *C. guthrieae* on MS growth medium supplemented with 4.4 µM BA/1.1 µM NAA. (**F**) Rooting response of *C. guthrieae* shoots on PGR-free full-strength MS medium after 8 weeks in culture. (**G**) *In vitro* rooted and greenhouse-acclimatised *C. obliquus* plantlets after 4 months

2.3.4. Effects of photoperiod on shoot multiplication

The regulation of photomorphogenetic processes of plant tissues in culture together with plant growth and quality is largely determined by light parameters provided in the culture environment (**ECONOMOU and READ, 1987**). Light duration is among the important environmental factors that affect plant growth and development in culture (**MURASHIGE, 1974**). The results of the morphogenetic parameters of *Cyrtanthus* shoots investigated at two different photoperiods are presented in **Table 2.2**. In all three species, the number of shoots produced per explant, and shoot length were higher in explants cultured under 16 h photoperiod. Shoot fresh and dry weights were, however, significantly higher in explants maintained under continuous light conditions suggesting an improved dry matter accumulation, possibly as a result of activated CK biosynthesis or its photo-oxidation to a desirable endogenous PGR ratio (**KAMADA et al. 1995; KOZAI and KUBOTA, 2005**). Consistent with these findings, was the significant increase in stem diameter, leaf area and number of leaves in *in vitro* potato plantlets cultured under long photoperiod and high PPF levels found by **KOZAI et al.** (**1995**). In the same experiment, longer photoperiods also led to an increase in fresh and dry weights with an enhanced root growth. In *Peperomia scandens*, **KUKLCZANKA et al.** (**1977**) reported improved shoot proliferation from leaf explants maintained under continuous light. The influence of photoperiod on various morphogenic parameters, however, vary from species to species. **DEBERGH and MAENE** (**1977**) reported an improved shoot multiplication frequency in shoot-tip cultures of *Pelargonium* species maintained under continuous light while a decrease in shoot number, length and quality was recorded in *Rhododendron* species under similar photoperiodic conditions (**ECONOMOU and READ, 1987**). Light is thought to exert these effects in plant tissue culture by either inducing the activation of CK biosynthesis and/or its degradation thereof, with the subsequent increase/decrease in the endogenous levels of CK (**TAPINGKAE and TAJI, 2000**).

Table 2.2. Effect of photoperiod on *in vitro* shoot morphogenesis of *Cyrtanthus* species

Species	Treatment	No. of shoots/explant		Shoot length (cm)		Fresh weight (mg)		Dry weight (mg)	
		16/8h (light/dark)	24h light	16/8h (light/dark)	24h light	16/8h (light/dark)	24h light	16/8h (light/dark)	24h light
C. contractus	10 µM TDZ	7.9 ± 0.9[b]	5.3 ± 0.6[a]	5.8 ± 0.8[b]	3.1 ± 0.4[a]	178.8 ± 2.7[a]	183.7 ± 1.6[a]	9.3 ± 1.1[a]	11.2 ± 0.7[a]
C. guthrieae	4.4 µM BA/1.1 µM NAA	15.4 ± 1.0[b]	11.5 ± 0.8[a]	5.8 ± 0.4[a]	4.7 ± 0.6[a]	123.4 ± 2.5[a]	131.1 ± 3.1[b]	10.1 ± 0.8[a]	13.3 ± 0.5[b]
C. obliquus	10 µM BA	4.0 ± 0.2[a]	3.1 ± 0.7[a]	4.7 ± 1.1[a]	3.2 ± 0.3[a]	87.1 ± 3.4[a]	93.4 ± 2.1[b]	6.7 ± 0.2[a]	10.2 ± 1.0[b]

Mean values ± standard error (n = 24) in the same row for each growth parameter with different letter(s) are significantly different ($P \leq 0.05$) based on LSD

2.3.5. Sucrose concentration on bulblet growth and multiplication

The trend observed in *C. contractus* and *C. guthrieae* showed a general increase in bulbils growth (diameter and fresh mass) with an increase in sucrose concentration up to 90 g l^{-1} (**Table 2.3**). As in other bulbous species such as *Narcissus*, *Cyrtanthus*, tulips, *Eucomis* and *Allium* (**HANKS et al. 1986; LE GUEN-LE SAOS et al., 2002; ANGULO et al., 2003; MORÁN et al., 2003; CHEESMAN et al., 2010**), sucrose concentration improves bulb formation and growth. **HANKS et al.** (**1986**) evaluated carbohydrate distribution in bulbs in relation to their ability to initiate adventitious bulbs and reported a positive correlation between the number of bulblets produced and the levels of carbohydrates. However, contrary to these observations, despite the high biomass accumulation at 90 g l^{-1} sucrose concentration in this study, bulblet regeneration significantly reduced in the three *Cyrtanthus* species. In general, shoot (not shown) and bulblet proliferation increased up to 30 g l^{-1} sucrose concentration after which the number of bulblets produced per explant decreased in a concentration-dependent pattern. One of the established roles of sucrose and other sugars in plants is to serve as signalling molecules (**ROLLAND et al., 2006; JAIN et al., 2007**). Plant growth critically depends on these signalling systems that provide information on the prevailing internal and external conditions. In interpreting their results on sucrose concentration-dependent dry matter accumulation in *Allium cepa* bulbs, **LE GUEN-LE SAOS et al.** (**2002**) attributed this characteristic to both the regulatory and growth promoting effects of sucrose. The greater mobilising and/or utilising ability of bulbs to sucrose could be associated with a higher content of available sucrose, glucose fructose and fructans which form major carbohydrate reserve in bulbs (**DARBYSHIRE and STEER, 1990; ERNST et al., 1998**). The general trade-off between bulblet proliferation and growth at sucrose concentration greater than 30 g l^{-1} might suggest a process-level regulation by sucrose, with a shift in assimilate partitioning towards bulb biomass accumulation. One school of thought postulates that the osmotic gradient generated by an increased sucrose concentration in the growth medium and tissue cells may elicit a shift in the growth and developmental program in a plant (**LE GUEN-LE SAOS et al., 2002; LEÓN and SHEEN, 2003; KOCH, 2004; SMEEKENS et al., 2010**).

Table 2.3. Effect of sucrose concentration on bulblet growth and proliferation of three *Cyrtanthus* species

Plant species	Sucrose (g l^{-1})	No. of bulblets per explant	Bulblet diameter (mm)	Bulblet fresh mass (mg)
C. contractus	0	0.24 ± 0.05^{a}	1.80 ± 0.17^{a}	107.6 ± 2.43^{a}
	10	0.52 ± 0.04^{b}	3.07 ± 0.15^{b}	156.5 ± 6.21^{b}
	30	2.18 ± 0.12^{c}	4.03 ± 0.12^{c}	204.4 ± 6.63^{c}
	60	0.50 ± 0.07^{b}	5.23 ± 0.09^{d}	447.1 ± 13.08^{e}
	90	0.10 ± 0.03^{a}	6.27 ± 0.19^{e}	593.1 ± 9.29^{f}
	120	0.08 ± 0.03^{a}	3.57 ± 0.27^{bc}	355.1 ± 16.34^{d}
C. guthrieae	0	1.77 ± 0.17^{d}	1.20 ± 0.17^{ab}	92.9 ± 3.32^{ab}
	10	1.31 ± 0.08^{c}	1.57 ± 0.12^{b}	110.7 ± 6.03^{b}
	30	1.81 ± 0.27^{d}	2.40 ± 0.21^{c}	163.3 ± 7.59^{c}
	60	0.61 ± 0.06^{b}	3.67 ± 0.35^{d}	241.7 ± 9.30^{d}
	90	0.34 ± 0.07^{ab}	2.38 ± 0.17^{c}	222.7 ± 5.49^{d}
	120	0.13 ± 0.03^{a}	0.80 ± 0.12^{b}	86.4 ± 4.32^{a}
C. obliquus	0	0.04 ± 0.02^{a}	1.27 ± 0.24^{a}	116.0 ± 3.79^{a}
	10	0.04 ± 0.02^{a}	1.90 ± 0.21^{b}	138.3 ± 2.03^{c}
	30	0.21 ± 0.07^{b}	2.23 ± 0.07^{b}	156.3 ± 5.55^{d}
	60	0.02 ± 0.01^{a}	0.97 ± 0.12^{a}	110.2 ± 6.43^{a}
	90	0.00 ± 0.00^{a}	ND	ND
	120	0.00 ± 0.00^{a}	ND	ND

Mean values ± standard error (n = 24) in the same column for each plant species with different letter(s) are significantly different ($P \leq 0.05$) based on DMRT
ND = not determined

In *C. obliquus*, bulblet diameter and fresh weight only increased up to a sucrose concentration of 30 g l^{-1} beyond which the two growth parameters significantly declined together with the number of bulblets produced per explant. At 90 g l^{-1} and higher, all *C. obliquus* plantlets gradually turned brown and died out. The response trend to sucrose concentration in *C. obliquus* attests to the assertion that response outputs vary as a function of genotype (**DEBERGH and MAENE, 1977; WU et al., 2012**) and that optimal levels for morphogenic responses are determined by interactive processes between intrinsic and extrinsic factors (**LEÓN and SHEEN, 2003**). The results indicate that the species has a lower sucrose requirements for

bulb growth than *C. contractus* and *C. guthrieae*. Growth was significantly reduced at sucrose concentrations of 90 g l^{-1} and above, in all three species, with most plantlets showing stunted growth. *Cyrtanthus contractus* and *C. guthrieae* share the same optimum sucrose levels for bulb development.

2.3.6. Indirect shoot organogenesis in *Cyrtanthus guthrieae*

In the absence of the possibility for developmental cell migration and due to the need for continuous organogenesis during their post-embryogenic development, plants maintain organ-forming cell files, the meristems (**FEHER et al., 2003**). The ontogenic program of plants is highly flexible owing to the reversibility of the differentiation state of somatic cells. The activity of meristematic cells are, therefore, dependent on environmental cues and other developmental factors such as PGRs. Plant growth regulators are the most likely candidates in the regulation of developmental switches. In this study, indirect shoot organogenesis was achieved from the callus of *C. guthrieae* through a combination of minute quantities of auxins (2,4-D and picloram) and CK (BA) (**Table 2.4**). Compared to 2,4-D, picloram initiated a high shoot induction efficiency, with gradual decrease as the concentration increased from 0.1 to 0.5 µM though not significantly. However, a marked significant decrease was recorded when picloram concentration was doubled from 0.5 to 1.0 µM, with the number of shoots produced dropping from 10.5 to 3.93 respectively. The highest number of shoots per callus (11.8) concurrently with the highest regeneration frequency (88%) were obtained from a combination of 0.1 µM picloram and 0.01 µM BA.

Table 2.4. Effect of different types and concentrations of plant growth regulators on shoot organogenic regeneration of *Cyrtanthus guthrieae* calli.

Plant growth regulators (µM)		Explant formed from callus	
2,4-D	BA	Regeneration frequency (%)	Explants/ callus
0.1		32	2.57 ± 0.33^{bcd}
0.5		44	2.10 ± 0.17^{abc}
1.0		27	0.80 ± 0.15^{ab}
0.1	0.01	38	0.57 ± 0.15^{a}
0.1	0.05	33	1.20 ± 0.06^{ab}
0.5	0.01	46	3.61 ± 0.25^{cde}
0.5	0.05	41	0.87 ± 0.18^{ab}
1.0	0.01	52	0.97 ± 0.18^{ab}
1.0	0.05	23	0.47 ± 0.09^{a}
Picloram			
0.1		86	10.73 ± 0.96^{h}
0.5		82	10.50 ± 1.35^{h}
1.0		56	3.93 ± 0.68^{de}
0.1	0.01	88	11.77 ± 0.83^{h}
0.1	0.05	84	7.07 ± 0.72^{g}
0.5	0.01	79	4.83 ± 0.32^{ef}
0.5	0.05	75	5.97 ± 0.83^{f}
1.0	0.01	49	3.34 ± 0.39^{cde}
1.0	0.05	51	4.60 ± 0.44^{ef}
Control (No PGR)		43	2.55 ± 0.11^{bcd}

Mean values ± standard error (n = 24) in the fourth column represent the number of shoots (≥5 mm long) per morphogenetic callus. Different letter(s) in the column indicate a significant difference ($P \leq 0.05$) based on DMRT

A significant number of studies have demonstrated that auxin appears to play important roles both in the induction of embryonic and organogenic development in cultured tissues and in the subsequent elaboration of proper morphogenesis (**ZIMMERMAN, 1993; SCHNEITZ et al., 1998; FEHER et al., 2003**). In evaluating the organogenic potential of *Cunila galioides*, **FRACARO and ECHEVERRIGARAY** (**2001**) reported favourable shoot organogenesis at extremely lower concentrations

of CK than that of auxin, findings of which coincides with the results obtained in this study. The positive influence of exogenously applied auxins, particularly 2,4-D on the induction of shoot organogenesis and embryogenesis have been elaborated in numerous studies (**LIN et al., 2011; BASKARAN and VAN STADEN, 2012**) as well as its interaction with other PGR such as CK (**SAGARE et al., 2000; BASKARAN and VAN STADEN, 2012**). Their organo- and morphogenic effects aslo vary with genotypes, with some types of PGR exerting significant positive effects in one species than the other as evidenced here in this study in the case of picloram and 2,4-D. Although the process of embryo and organ induction from cells in culture is not well understood (**ZIMMERMAN, 1993**), in light of these results, it is tempting to speculate that auxin stimulated the pro-embryogenic masses within the cell cultures to synthesise all the gene products necessary to initiate cell proliferation and differentiation as modulated by CK. Whether auxin treatment stimulates more than just cell proliferation as a prerequisite to organogenesis or embryogenesis remains unclear (**TSUKAYA and BEEMSTER, 2006**).

When the regenerated microshoots were transferred to the shoot proliferation medium (4.4 µM BA/1.1 µM NAA), shoot multiplication increased rapidly with continuous production of callus at the base of shoot clusters (**Figure 2.4**). In terms of shoot multiplication rates, significantly higher shoot numbers were obtained via the indirect shoot organogenesis experiment compared to the previously described protocol (**Section 3.2.3**) for *C. guthrieae*. Through indirect organogenesis, multiple shoots developed from the calli that continued to be produced by the multiplying shoots in the proliferation medium. However, in contrast, calli maintained directly on shoot multiplication medium without prior culturing on either 2,4-D or picloram and BA, multiplied more callus and took longer (6 weeks) to produce microshoots. This phenomenon further affirms the significance of a fine-tuned delicate balance of endogenously applied PGR on their stimulatory effects on the process of shoot organogenesis.

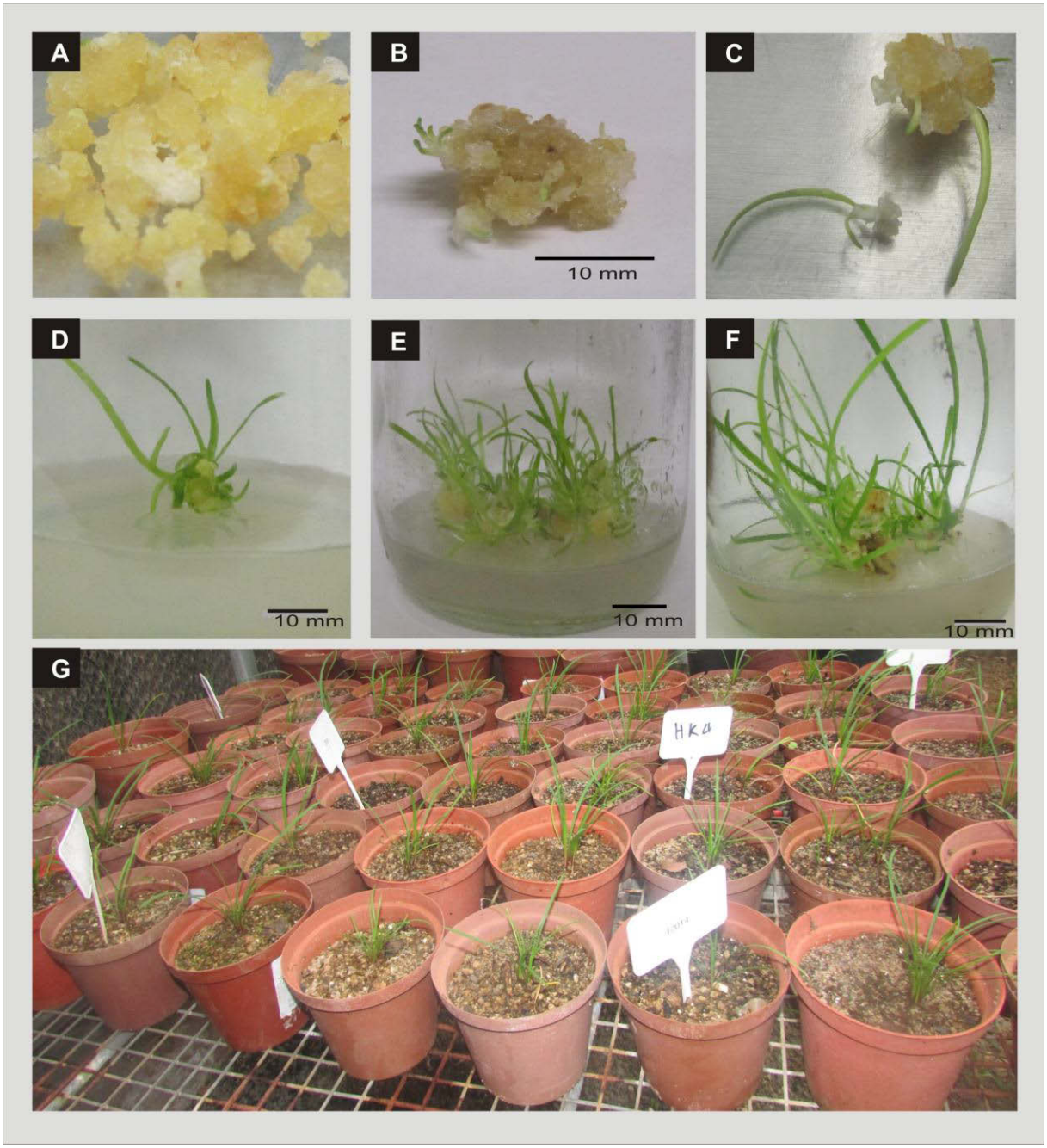


Figure 2.4. Callus and callus-derived *C. guthrieae* plantlets. (**A**) Callus. (**B**) Multiple microshoots developing on calli. **C.** Shoots developed through organogenesis from *C. guthrieae* callus. (**D**) Plantlet development on proliferation media after 4 weeks. (**E**) Shoot proliferation after 6 weeks on proliferation medium. (**F**). Plantlets maintained in proliferation medium 8 weeks after the second subculturing. (**G**) Rooted and acclimatised callus-derived plantlets after 4 weeks in the greenhouse

2.3.7. Rooting and acclimatisation

In vitro regenerated shoots from the optimised shoot multiplication experiments rooted effectively after four weeks of culture when transferred to full- and half-

strength MS media without PGRs (**Figure 2.3F**). The competent *ex vitro* acclimatisation of *in vitro* regenerated plants within the shortest possible time is a critical step in the conservation of any plant species. Based on the results from the multiplication experiments, regenerated shoots were rooted individually on half- and full-strength MS medium without PGRs. **Figure 2.5** shows the effects of the growth media on the rooting frequency and root growth parameters. Mean number of roots and root lengths were slightly higher in shoots cultured on full-strength MS in all three species although not significantly from those maintained on half strength MS. The regeneration frequency was more than 75% for all three *Cyrtanthus* species on both media, suggesting that either of the two media treatments can be used for *in vitro* rooting of the species. Agar was carefully washed off the *in vitro* rooted plantlets and they acclimatised and established successfully in the greenhouse (**Figure 2.3G**), with a more than 95% survival rate and no observable morphological abnormalities on all species. The high rooting frequency and number of roots per shoot produced on PGR-free medium in the three *Cyrtanthus* species, with subsequent high survival rate upon *ex vitro* acclimatisation, make these regeneration protocols particularly attractive economically in that they reduce labour and production costs.

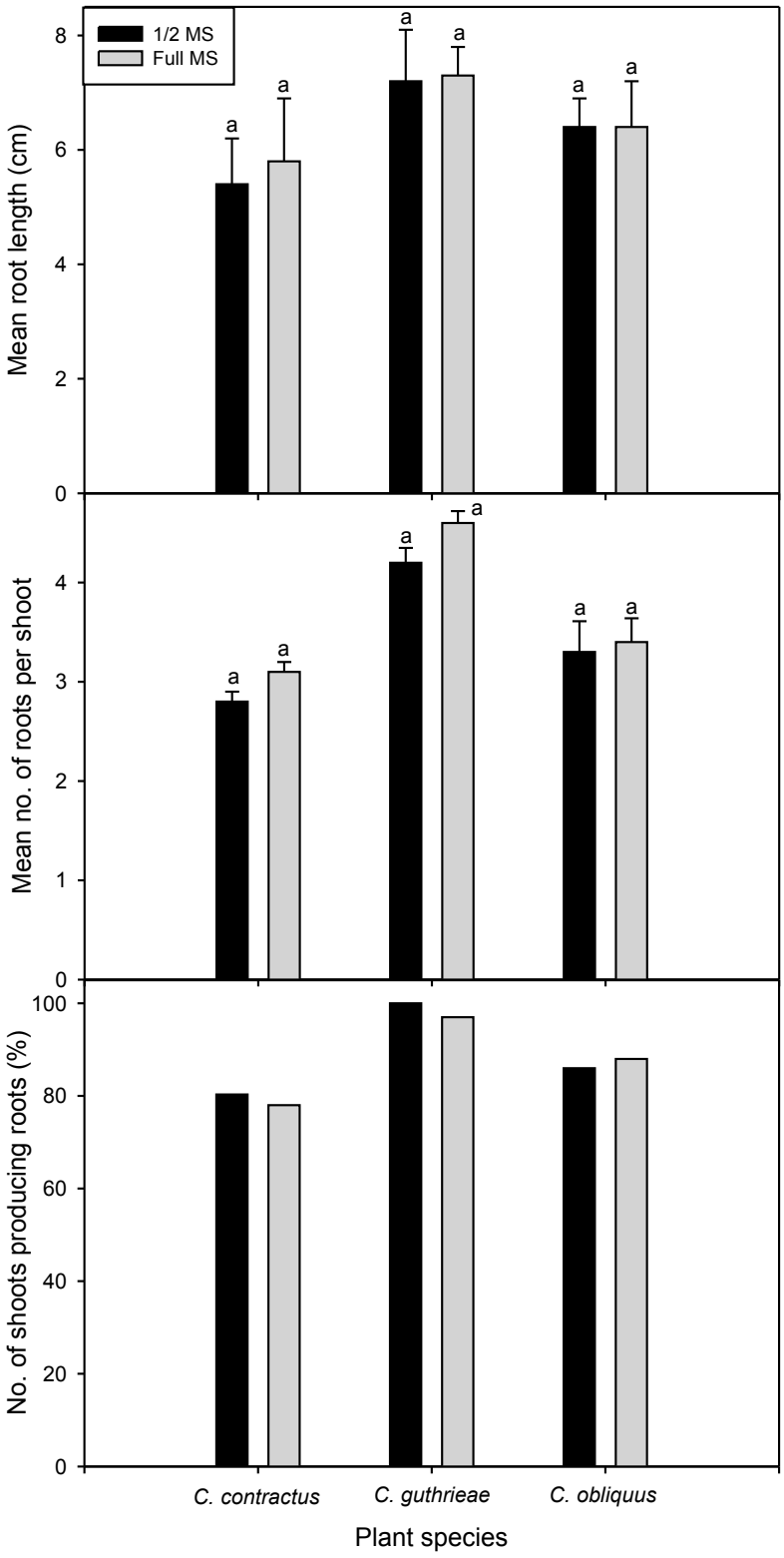


Figure 2.5. Effect of half-strength and full-strength MS media on rooting and root parameters of three *Cyrtanthus* species. Error bars with similar letters for each species indicate insignificant difference ($P \leq 0.05$) based on the *t*-test

2.4. Conclusions

As demonstrated by several experimental observations, including results of this study, the differentiated fate of plant cells, dependent on the positional information and developmental signals, can be altered desirably through manipulation of *in vitro* culture conditions. The successful *in vitro* propagation systems described here provide effective means for the conservation and rapid clonal multiplication, within reasonably short time frames, of the three threatened *Cyrtanthus* species. Owing to the declining populations of *Cyrtanthus* species in the wild, the developed micropropagation procedures can be used effectively for their rapid multiplication to meet commercial demands and in other conservation programmes. Manipulation of the medium composition (PGR) and culture environmental factors was effective in the development of micropropagation protocols for these species. The high number of plantlets generated through the proposed procedures together with the increased multiplication rate and cost-effective, easy acclimatisation process makes these protocols highly lucrative and represent excellent alternatives to conventional horticultural propagation methods.

CHAPTER 3

Physiological and metabolic response to *in vitro* culture manipulation in *Cyrtanthus* species

3.1. Osmotic and salinity stress on *in vitro* secondary metabolism of *Cyrtanthus* regenerants

3.1.1. Introduction

The central aspects of plant survival in a dynamic environment are the identification of evolutionary forces, the plant's inherent mechanism to detect these, respond and the subsequent ways in which this biodiversity is maintained and/or perpetuated (**NCUBE et al., 2012**). As organisms that lack the ability to move, plants often encounter a multitude of persistent (or episodes of) sub-optimal environmental conditions in their habitats. In response to this, the flexibility of their normal metabolism allows the development of responses to such environmental changes, which fluctuate regularly and predictably over daily and seasonal cycles (**BRAY, 1997**). Thus, not all deviations of environmental conditions and their optimal states constitute stress to plants. An altered physiological condition imposed on regular metabolic patterns caused by factors that tilt equilibrium beyond the normal fluctuating boundaries would, thus constitutes a stress parameter. Several of these factors qualitatively affect the plant's metabolic processes through their effects on plant development, growth rates and partitioning of assimilates into vital metabolites and are often linked to mortality events (**SALA et al., 2012**).

The metabolic alterations caused by abiotic stress factors such as drought, temperature, salinity and pH in plants range from synthesis of limited quantities of specialised metabolites to large shifts in primary metabolite composition together with many other physiological conformational changes. Among the environmental stress factors confronting plants on a more regular basis, drought has become the focus of many research objectives (**MENDOZA et al., 1996; ISHITANI et al., 1997; SALA et al., 2012**). Osmotic stress perception and signalling in plants, is translated into biochemical reactions, metabolic and physiological adjustments, with

consequent changes in the progression of growth and development (**BOHNERT and SHEVELEVA, 1998**). Relevant to the aspect of stress-mediated adjustments of metabolism, is the recognition that stress responses are elicited through several pathways and that these pathways are cross-linked (**HIRT, 1997; BOHNERT and SHEVELEVA, 1998; APEL and HIRT, 2004**). If the highly integrated metabolic system of processes is assumed to operate at almost optimal levels under normal conditions, then under stressful conditions certain aspects of intermediary metabolism and the associated precursor pools are likely to be affected more than others. In energy terms, such metabolic adjustment processes always exert heavy demands on the plant's often limited resource pool. The process of photosynthesis provides the carbon and nitrogen precursor molecules to drive these diverse complex metabolic processes. However, in most plant species, factors that affect water supply such as salinity and osmotic stress alter the ratio between the rate of light absorption and photosynthetic capacity, leading to photoinhibition (**ANGELOPOULOS et al., 1996; MORIANA et al., 2002**). These changes set in motion a series of metabolic events originating from changes in the concentration of leaf-internal CO_2 and consequently affecting the carbon reduction cycle, light reactions and energy charge (**BOHNERT and JENSEN, 1996; INGRAM and BARTELS, 1996; BOHNERT and SHEVELEVA, 1998**). Low internal CO_2 concentrations result in a reduction of the oxidised $NADP^+$ pool as an electron acceptor and most of the absorbed light energy is then diverted to molecular oxygen. This then creates an increased level of reactive oxygen species (ROS) (**SMIRNOFF, 1993; DAT et al., 2000**). The highly reactive nature of ROS is associated with the acute disruption of the plant's normal metabolism and damage to cellular structures (**HENLE and LINN, 1997**). For continued survival and growth, plants will thus adapt their physiology and metabolism to offset these devastating effects. Eventually, carbon and nitrogen allocation and storage is readjusted and reactions that lead to the consumption of reducing power become favoured.

Plants adjust to stress-mediated changes in a number of ways. Proline occupies a central place in this metabolism and is connected to various other pathways (**ASHRAF and FOOLAD, 2007; VERSLUES and SHARMA, 2010**). Proline accumulation primarily occurs in response to stresses that dehydrate plant tissues such as drought, salinity and freezing (**VERSLUES et al., 2006)**. Under conditions of

water deprivation or extreme salinity, proline accumulation serves as defence against osmotic challenge by acting as a compatible solute to increase cellular osmolarity thereby avoiding deleteriously high ionic strength (**DELAUNEY and VERMA, 1993**). Besides its stabilising effects on the cellular structure, proline metabolism also has roles in redox buffering, energy transfer, protecting enzymes, protein and cellular degradation, storage of carbon and nitrogen for rapid growth after stress and detoxification of free radicals by forming long-lived adducts with them (**SMIRNOFF and CUMBES, 1989; HARE and CRESS, 1997**). The surge in proline biosynthesis in response to abiotic stress in many plant species makes it worthwhile to consider how this metabolite may influence other pathways, particularly those that are likely to have their own functions in plant stress responses. The biochemical logic of stress-induced proline biosynthesis, as proposed by **SHETTY et al.** (**2004**) is coupled to the pentose-phosphate pathway, driving the synthesis of $NADPH_2$ and sugar phosphates for anabolic pathways, including phenolic and antioxidant response pathways, while simultaneously providing reducing equivalents needed for mitochondrial oxidative phosphorylation in the form of proline as an alternative to NADH. The low molecular weight secondary metabolites, phenolic compounds are produced by plants as part of a complex antioxidant system to cope with ROS.

Although plant species vary in their sensitivity and response to the decrease in water potential caused by drought, low temperature, or high salinity, it may be assumed that all plants have an encoded capability for stress perception, signalling, and response. The dynamic and multifaceted nature of metabolic adjustments in response to unfavourable conditions, not only depends on the type and magnitude of stress, but also on the plant species involved. In keeping with this view, the question is: can the manipulation of stress parameters within a plant's growing environment provide insight into the stress-related metabolic adjustments of a given species? *Cyrtanthus* (Amaryllidaceae) is an important genus of monocotyledonous plants whose bulbs and leaves are highly sought after for medicinal purposes in South Africa. Due to their valuable use in traditional medicine and the interests in the search for natural plant-derived and novel compounds, understanding their metabolic response to various environmental factors thus becomes a crucial aspect. In an attempt to further our understanding of the species, *in vitro*-cultured *C. contractus*

and *C. guthrieae* plantlets (**Section 2.2.2**, Chapter 2) were used for the mechanistic model study of their metabolic response to salinity and osmotic stress.

3.1.2. Materials and methods

3.1.2.1. Plant material and growth conditions

The unrooted *in vitro*-regenerated *C. contractus* and *C. guthrieae* plantlets obtained from the optimised micropropagation protocols (**Section 2.2.2**, Chapter 2) were maintained on PGR-free MS medium for 8 weeks before sub-culturing in different salt and osmotic stress-inducing media at 25 ± 2 °C with a 16 h photoperiod. Unless specified otherwise, all culture environmental conditions were as described in **Section 2.2.2**.

3.1.2.2. Osmotic and salinity stress treatments

Salt stress levels were induced on solid MS medium by adding 50, 100, 150 and 300 mM NaCl while osmotic stress levels were induced using polyethelyneglycol (PEG) 6000 (Merck, Germany) at 50, 100, and 150 g l^{-1} of medium. A control treatment with MS medium only was also included. In screw cap culture jars (110 mm × 60 mm, approximately 300 ml volume), plantlets maintained on medium described in **Section 3.2.2.1** were cultured on 30 ml of the different stress-induced media treatments and maintained at 25 ± 2 °C under 16 h photoperiod. Each treatment had 30 plantlets replicated twice. At three, four and five weeks from initial plant culture, samples from each stress treatment were taken for various biochemical analyses.

3.1.2.3. Determination of proline content

Free proline content was determined using the ninhydrin reagent method as described by **BATES et al.** (**1973**) with slight modifications. Fresh whole plantlet samples (1 g) from each treatment were homogenised in 10 ml of a 3% (w/v) aqueous sulfosalicylic acid solution with the addition of a few grains of acid-washed quartz sand (BDH Chemicals, England) in a pestle and mortar. The homogenate was filtered through two layers of glass-fibre (Schleicher and Schüll, GF6, Germany), and

the clear filtrate was then used in the assay. One millilitre of ninhydrin reagent (2.5 g 100 ml^{-1} of a solution containing glacial acetic acid, distilled water and 85% ortho-phosphoric acid at a ratio of 6:3:1) were added to 1 ml of the filtrate in test tubes. The closed test tubes with the reaction mixture were kept in a boiling water bath for 1 h, and the reaction terminated in a water bath for 5 min at room temperature. Absorbance was measured at 546 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia) against a blank that contained 3% (w/v) aqueous sulfosalicylic acid instead of sample extract. A freshly prepared stock of proline (Sigma-Aldrich) solution (0.1 mg ml^{-1} in 3% (w/v) aqueous sulfosalicylic acid) was used for the standard curve. Proline concentrations were determined from a standard curve and calculated on a fresh weight basis (µmol proline g^{-1} FW).

3.1.2.4. Preparation of extracts for polyphenol determination

Phenolic compounds were extracted from plant material as described by **MAKKAR** (**1999**). Dried whole plantlet samples from each treatment (1 g) were extracted with 10 ml of 50% (v/v) aqueous methanol by sonication on ice for 20 min. The extracts were then filtered under vacuum through Whatman No. 1 filter paper and used in the assays outlined below (**Sections 3.1.2.5** to **3.1.2.7**).

3.1.2.5. Total polyphenol content

The amount of total phenolic compounds in plant samples were determined using the Folin Ciocalteu (Folin C) assay for total phenolics as described by **MAKKAR** (**1999**) with slight modifications. In triplicate, 50 µl of each treatment extract were transferred into test tubes and the volume made up to 1 ml with distilled water (950 µl). Folin C phenol reagent (500 µl, 1 N) and 2% sodium carbonate (2.5 ml) were added to each dilute sample. A blank that contained 50% aqueous methanol instead of sample extract was also prepared. After incubation for 40 min at room temperature absorbance was read at 725 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia). A gallic acid (Sigma-Aldrich) standard curve was prepared from a freshly made stock solution (0.1 mg ml^{-1}) in distilled water. Total phenolic contents were expressed as gallic acid equivalents (GAE) per g dry weight

(DW) as determined from the standard curve. Each sample extract had three replicates.

3.1.2.6. Flavonoid content

The vanillin assay, as described by **HAGERMAN** (**2002**) was used to determine flavonoid content from plant samples. Extracts (50 µl), were made up to 1 ml with methanol in test tubes before adding 2.5 ml methanolic-HCl (95:5, v/v) and 2.5 ml vanillin reagent (1 g 100 ml^{-1} acetic acid). Similar preparations of a blank that contained methanol instead of plant extracts were made. After 20 min incubation at room temperature, absorbance was read at 500 nm using a UV-vis spectrophotometer. A catechin standard curve was prepared from 1 mg ml^{-1} catechin (Sigma-Aldrich) stock solution in methanol. The flavonoid content was expressed as µg catechin equivalents (CTE) per g DW. Each sample extract had three replicates.

3.1.2.7. Proanthocyanidins (condensed tannins)

The butanol-HCl assay (**PORTER et al., 1985**) was used to quantify proanthocyanidin content. Three millilitres of butanol-HCl reagent (95:5, v/v) were added to 500 µl of each extract, followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test combination was mixed with a vortex and placed in a boiling water bath for 60 min. Absorbance was then read at 550 nm using a UV-vis spectrophotometer against a blank prepared in a similar way but without heating. Each sample extract had three replicates. Proanthocyanidins (mg LCE g^{-1} DW) were calculated as leucocyanidin equivalents (LCE) using the formula developed by **PORTER et al.** (**1985**):

Condensed tannin (%) = ($A_{550\ nm}$ x 78.26 x Dilution factor) / (% dry matter)

where $A_{550\ nm}$ is the absorbance of the extracts at 550 nm, 78.26 is the molecular weight of leucocyanidin and the dilution factor was 0.5 ml/ volume of extract taken. The formula assumes that the effective $E^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460.

3.1.2.8. Proline dehydrogenase (EC 1.5.99.8) enzyme activity

Enzyme extractions were carried out in an ice bath. Proline dehydrogenase (PDH) (EC 1.5.99.8) was extracted from fresh plantlet samples (1 g) by homogenising in 2 ml of chilled potassium phosphate buffer (0.1 M, pH 7.8) containing 0.5% (v/v) Triton X-100 and 1 % (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The resulting slurry was filtered through two layers of cheese cloth and the filtrate was centrifuged for 20 min at 10 000 *g* at 4 °C. The supernatants were desalted on a Sephadex G-25 column (Pharmacia AB, Sweden) and eluted with 0.05 M Tris-HCl (pH, 7.4) buffer containing 10% glycerol. The extracts were used immediately for the assay. To determine the activity of PDH, the reaction mixture contained L-proline (Sigma-Aldrich) (15 mM), cytochrome *c* (Sigma-Aldrich) (0.01 mM), phosphate buffer (0.1 M, pH 7.4), 0.5% (v/v) Triton X-100, and the enzyme extract (0.2 ml) in a total volume of 1 ml. The reaction mixture was incubated at 37 °C for 30 min and the reaction terminated by adding 1 ml of 10% trichloroacetic acid (TCA). The Δ^1-pyrroline-5-carboxylic acid (P5C) formed was measured by adding 0.5% o-aminobenzaldehyde in 95% aqueous ethanol. The mixture was further incubated at 37 °C for 10 min, centrifuged at 10 000 *g* and absorbance of the coloured supernatant complex read at 440 nm. Enzyme activity was calculated using a molar extinction coefficient of 2.71 x 103 min^{-1} cm^{-1} for P5C and from the standard curve using P5C (Sigma-Aldrich) and expressed as nmol P5C min^{-1} mg^{-1} protein. Protein content was measured following the method of **BRADFORD** (**1976**) as described in **Section 3.2.2.5** with bovine serum albumin (BSA) as the standard.

3.1.2.9. Photosynthetic pigments

The quantitative determination of chlorophyll (Chl *a* and *b*) and carotenoids were as described by **LICHTENTHALER** (**1987**). Fresh plantlet samples (0.2 g) were homogenised in 5 ml acetone with addition of a few grains of acid-washed quartz sand. The homogenate was filtered through Whatman No. 1 filter paper and centrifuged for 5 min at 5 000 *g* at room temperature. The absorbance of the resultant filtrate was measured at 470, 645, and 662 nm using a UV-vis spectrophotometer. The pigment contents were calculated from the following formulae and expressed as µg g^{-1} FW.

Chlorophyll *a* (C_a) = $11.24A_{662} – 2.04A_{645}$

Chlorophyll *b* (C_b) = $20.13A_{645} – 4.19A_{662}$

Total chlorophyll = $7.05A_{662} + 18.09A_{645}$

Carotenoids = $(1000A_{470} – 1.90C_a – 63.14C_b)/214$

3.1.2.10. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software for Windows (IBM SPSS®, version 21.0, Chicago, USA). Where there were significant differences ($P \leq 0.05$), the means were further separated using Duncan's multiple range test (DMRT) and/or Least Significant Difference (LSD).

3.1.3. Results and discussion

3.1.3.1. Proline biosynthesis in relation to stress factor effects

The levels of proline content measured at intervals of three, four and five weeks from initial plantlet culture were found to increase in a stress-dependent pattern (**Figure 3. 1**). Although most of the stress treatment levels did not differ significantly in proline content with the control at three weeks, proline levels increased significantly from four to five weeks of culture duration in both species. The highest proline concentration (9.98 µmol g^{-1} FW) was elicited in *C. contractus* at 300 µM NaCl after five weeks. Compared to the control plants during the same period, this amount represents more than 5-fold increase in proline accumulation. High salt stress levels of 150 and 300 mM appeared to trigger high proline biosynthesis, with both treatments exhibiting markedly higher levels with an increase in culture duration in both species. In *C. guthrieae*, all PEG treatments and the lower levels of salt stress (50 and 100 mM) did not show major differences from the control between three and four weeks of culture.

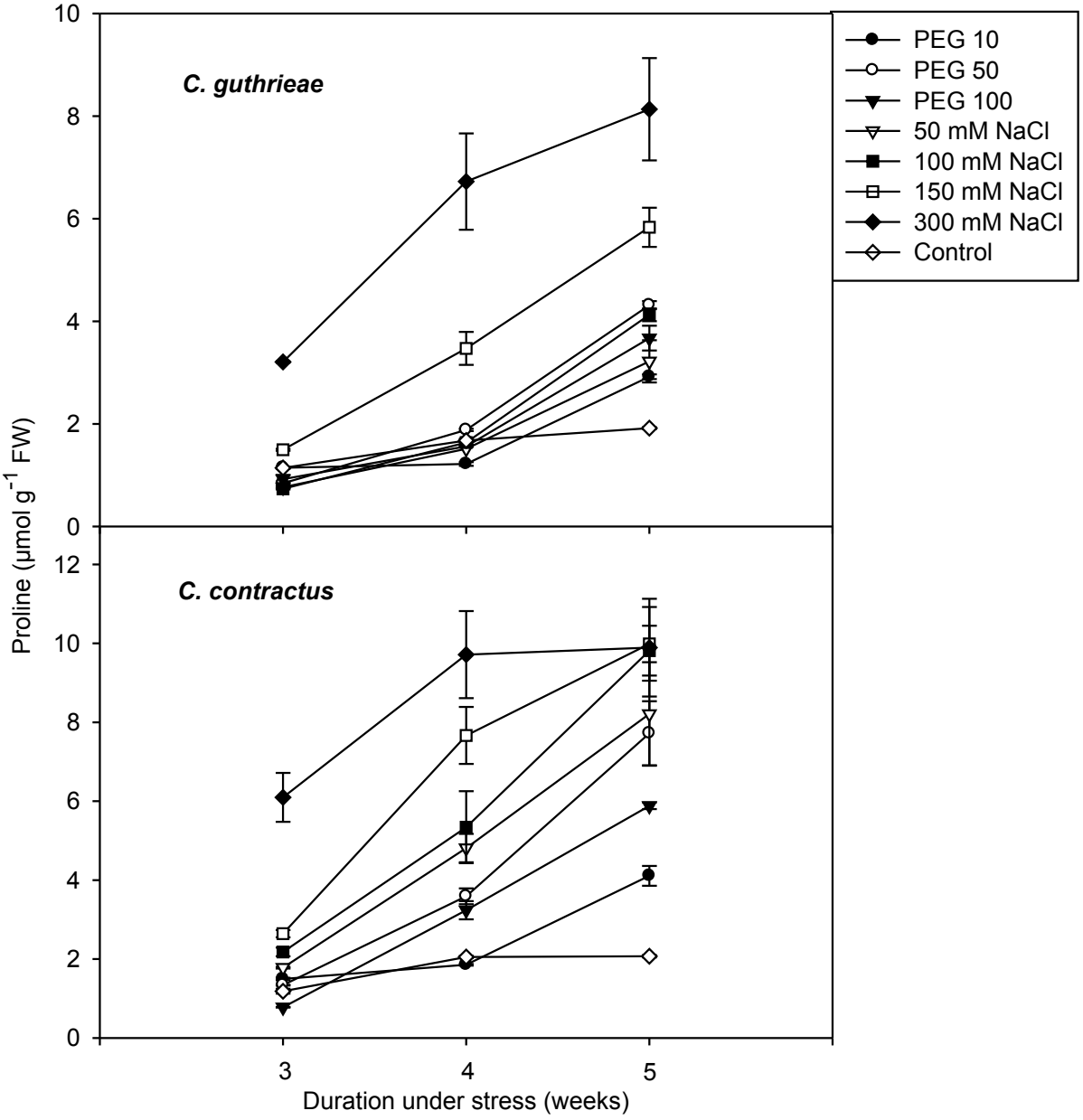


Figure 3.1. Free proline content (µmol proline g^{-1} FW) in response to different levels and duration of salinity and osmotic stress in *C. contractus* and *C. guthrieae*

Drought and salinity affect the uptake and water conductance in plants. Such environmental factors that affect water supply lead to changes in stomatal opening and set in motion a series of biochemical processes which consequently affect the carbon reduction cycle. The large accumulation of proline that occurred with the increased water and salt stress in this study may be explained in part by the basic chemical properties of this compound. Proline is one of the most water soluble compatible solutes that exist mostly in a zwitterionic state having both weak negative

and positive charges at the carboxylic acid and nitrogen groups, respectively (**VERSLUES and SHARMA, 2010**). This property makes proline a worthwhile and preferred compound accumulated by most plant species and other organisms to adjust cellular osmolarity (**YANCEY, 2005**). It was estimated, for example, in the root apex of maize grown at low water potential that proline accumulation may account for approximately 45% of the total osmotic adjustment (**VOETBERG and SHARP, 1991**). Salt, on the other hand, presents osmotic and ionic components of stress in plants with two possible phases of impacts (primary and secondary respectively), where plants react to each component at different times (**SHAVRUKOV, 2013**). The osmotic component of salinity stress occurs immediately when roots come into contact with solutions containing unfavourably high concentrations of salts in the growth medium and plants need to adjust for water potential and turgor to achieve homeostasis (**JAMES et al., 2008**). The ionic stress component, as a second phase, gradually becomes more severe at a later stage because the concentration of Na^+ have to reach a certain toxic level in cell protoplasts of shoots and this process requires some time (**MUNNS, 2002; JAMES et al., 2008; MUNNS and TESTER, 2008**). Considering that both phases of salinity stress responses require cellular osmotic adjustments, it is likely that high proline accumulation recorded at high salt concentration treatments (150 and 300 mM) may serve this protective role. Following this logic, high osmotic and ionic potential would thus translate to a high accumulation of compatible osmolytes. Apparent correlations between high proline and the severity of stress have led to some conclusions that the metabolite is an adaptive response (**HARE and CRESS, 1997**). Although, many researchers have reported positive correlations between proline accumulation and stress factor effects in different plant species (**STINES et al., 1999; ROSHANDEL and FLOWERS, 2009**) others have challenged the value of proline accumulation as a means of alleviating cellular osmotic stress damage (**VERSLUES and SHARMA, 2010**). However, the validity of many of these arguments rests on the presence of a cytoplasmic pool of free proline of sufficient size to provide a pronounced osmotic influence.

Abiotic factors that constrain water availability in plants often lead to extensive accumulation of ROS during stress and proline acts as a scavenging molecule that reacts and detoxifies ROS (**SMIRNOFF and CUMBES, 1989**). The ability of cells to

perceive and precisely respond to environmental changes forms the basis of cellular signalling. A positive correlation between proline and phenolic compound accumulation with an increase in salt and osmotic stress severity and duration observed in this study, suggests a cross link between these signalling pathways. Thus, these metabolites may have an important role in the acclimation process to these stress effects. Proline, in particular has one of its suggested roles as a signalling molecule in which its transport between different plant parts serves as a metabolic signal (**HARE and CRESS, 1997; KISHOR et al., 2005; VERSLUES and SHARMA, 2010**). Stress-mediated proline biosynthesis consumes high amounts of NADPH reductants, which may assist in the restoration of the terminal electron acceptor of the photosynthetic electron transport chain (**LEHMANN et al., 2010; VERSLUES and SHARMA, 2010**). In addition, an increased $NADP^+$/NADPH ratio that results from proline biosynthesis is likely to enhance the activity of the oxidative pentose phosphate pathway (**SHETTY, 2004**), which would then provide precursors to support the demand for increased secondary metabolite production during stress. The high levels of different classes of phenolic compounds observed in this study (**Figure 3.2**) could perhaps partly be explained on this basis.

3.1.3.2. Proline dehydrogenase activity

To elucidate the mechanism of proline metabolism during saline and water stress, the activity of the enzyme proline dehydrogenase (PDH) was investigated. Proline dehydrogenase is the first enzyme of the proline catabolic pathway which catalyses the oxidation of proline to Δ^1-pyrroline-5-carboxylate (P5C) in the mitochondria. A significant decline in the activity of this enzyme was observed in both species during stress conditions (**Table 3.1**). The degree of decline was dependent on the severity and duration of the stress treatment. In the absence of stress (controls), there was no significant difference in the PDH activity in both species. High salinity stress (300 mM) presented the lowest enzyme activity, with magnitudes of 27- and 51-fold decreases at 5 weeks in *C. contractus* and *C. guthrieae* respectively compared to the controls. As presented above (**Section 3.1.3.1**), the levels of proline registered their highest concentrations in the same treatment (300 mM NaCl). The level of free proline is regulated by the ratio of the rates of its biosynthesis and degradation. The transcriptional upregulation of proline biosynthesis and downregulation of its

degradation are thought to control proline accumulation during stress (**STINES et al., 1999; ROSHANDEL and FLOWERS, 2009**). Downregulation of PDH could be interpreted as one of the control mechanisms to allow proline accumulation during stress. In an integrated transcriptome and metabolome study with grape, proline levels during water deficit and salinity stress have been linked with changes in gene expression for some of the enzymes of proline metabolism (**CRAMER et al., 2007**). A concomitant linkage between changes in proline content and the enzyme activity in this study complement and extend previous research findings. While the findings of this study are consistent with those of previous workers with different plant species (**DELAUNEY and VERMA, 1993; MADAN et al., 1995; INGRAM and BARTELS, 1996**), upregulation of PDH concurrently with the high accumulation of proline have also been reported in similar stress regimes (**KOHL et al., 1991; KAPLAN et al., 2007**). Given these contrasting sets of evidences, the signalling and regulation mechanisms of proline metabolism thus warrant further elucidation. Be that as it may, it remains logical to assume that the strong negative correlation between proline accumulation and the enzyme responsible for its catabolism serves to maintain the high levels of this metabolite during stress. The decrease in the PDH activity suggests a reduction in proline degradation while allowing accumulation of high levels through biosynthesis in the stress conditions. The very low activity of this enzyme recorded at the highest salinity stress (300 mM) compared to the control plants points to the accumulation of proline as a symptom of metabolic perturbation in suboptimal plant growth environments. In this regard, it would seem reasonable that a large reduction in enzyme activity parallel to the large increase in proline biosynthesis could be a function of a basal and acquired tolerance mechanism.

Table 3.1. Effect of varying levels and duration of salinity and osmotic stress on proline dehydrogenase activity of *in vitro* regenerants of *C. contractus* and *C. guthrieae*

Stress treatment	*C. contractus* (nmol P5C min^{-1} mg^{-1} protein)			*C. guthrieae* (nmol P5C min^{-1} mg^{-1} protein)		
	Week 3	Week 4	Week 5	Week 3	Week 4	Week 5
PEG 10	6.8 ± 0.8^{b1}	6.1 ± 0.6^{b1}	3.4 ± 0.2^{b2}	4.4 ± 0.5^{ab1}	4.1 ± 0.2^{a1}	2.4 ± 0.4^{bc2}
PEG 50	6.5 ± 0.1^{b1}	4.8 ± 0.4^{c2}	2.1 ± 0.3^{b3}	4.3 ± 0.1^{ab1}	4.8 ± 0.3^{a1}	3.3 ± 0.2^{b2}
PEG 100	4.2 ± 0.2^{c1}	2.1 ± 0.3^{c2}	1.8 ± 0.5^{bc2}	4.3 ± 0.4^{ab1}	4.1 ± 0.2^{a1}	3.5 ± 0.3^{b1}
50 mM NaCl	4.3 ± 0.3^{c1}	3.2 ± 0.3^{c2}	1.9 ± 0.4^{bc3}	3.5 ± 0.1^{b1}	2.9 ± 0.8^{b1}	1.7 ± 0.1^{c2}
100 mM NaCl	2.1 ± 0.3^{d1}	1.1 ± 0.0^{cd2}	0.7 ± 0.0^{c2}	2.1 ± 0.6^{bc1}	1.9 ± 0.1^{c1}	1.1 ± 0.0^{cd2}
150 mM NaCl	2.3 ± 0.1^{d1}	0.9 ± 0.1^{d2}	0.7 ± 0.3^{c2}	1.3 ± 0.1^{c1}	0.4 ± 0.1^{d2}	0.4 ± 0.1^{d2}
300 mM NaCl	0.8 ± 0.1^{e1}	0.3 ± 0.0^{d2}	0.3 ± 0.1^{c2}	0.2 ± 0.0^{cd1}	0.1 ± 0.0^{d2}	0.1 ± 0.0^{d2}
Control	8.1 ± 0.7^{a1}	8.3 ± 0.3^{a1}	8.8 ± 0.2^{a1}	5.3 ± 0.8^{a1}	4.9 ± 0.3^{a1}	5.1 ± 0.7^{a1}

Mean values ± standard error (n = 3) in the same column and row with different letter(s) and numeric number(s) respectively for each species are significantly different ($P \leq 0.05$) according to DMRT

3.1.3.3. Salinity and osmotic stress effects on phenolic content

The trend in the accumulation of total phenolic compounds and proanthocyanidin content in *C. contractus* followed a somewhat similar pattern as that of proline (**Figure 3.2A, E**). However, the highest levels of polyphenols were recorded with 150 mM salt stress compared to the 300 mM in the case of proline. Total phenolic compounds produced in *C. contractus* at 150 mM salt stress treatment was consistently higher (> 140 mg GAE g^{-1}) than for all other stress factor levels after three weeks of culture, with a subsequent insignificant increase up to the fifth week. Although total phenolic levels were shown to increase in a stress-dependent pattern with an increase in culture duration, the increase was not as sharp as that exhibited by proline. In contrast with *C. contractus*, total polyphenol levels in *C. guthrieae* increased sharply from week three to week four in culture, with very slight and mostly insignificant increases between the fourth and fifth week in all stress factor levels (**Figure 3.2F**). Polyethylene glycol (PEG 150) resulted in the second highest levels of total phenolic compounds after 150 mM NaCl compared to the 100 mM NaCl in *C. contractus*. Apart from these trends, proanthocyanidin accumulation exhibited an almost similar characteristic pattern in both species (**Figure. 3.2A & B**). As with *C. contractus*, total phenolic levels in *C. guthrieae* across the entire stress duration were also high at salt stress level of 150 mM. On a treatment by treatment comparison, *C. guthrieae* generally recorded high concentrations of total phenolics in most treatments at five weeks compared to *C. contractus*. Generally, flavonoid content (**Figure 3.2C & D**) in most treatments, increased between three and four weeks before either levelling out or dropping down in the fifth week.

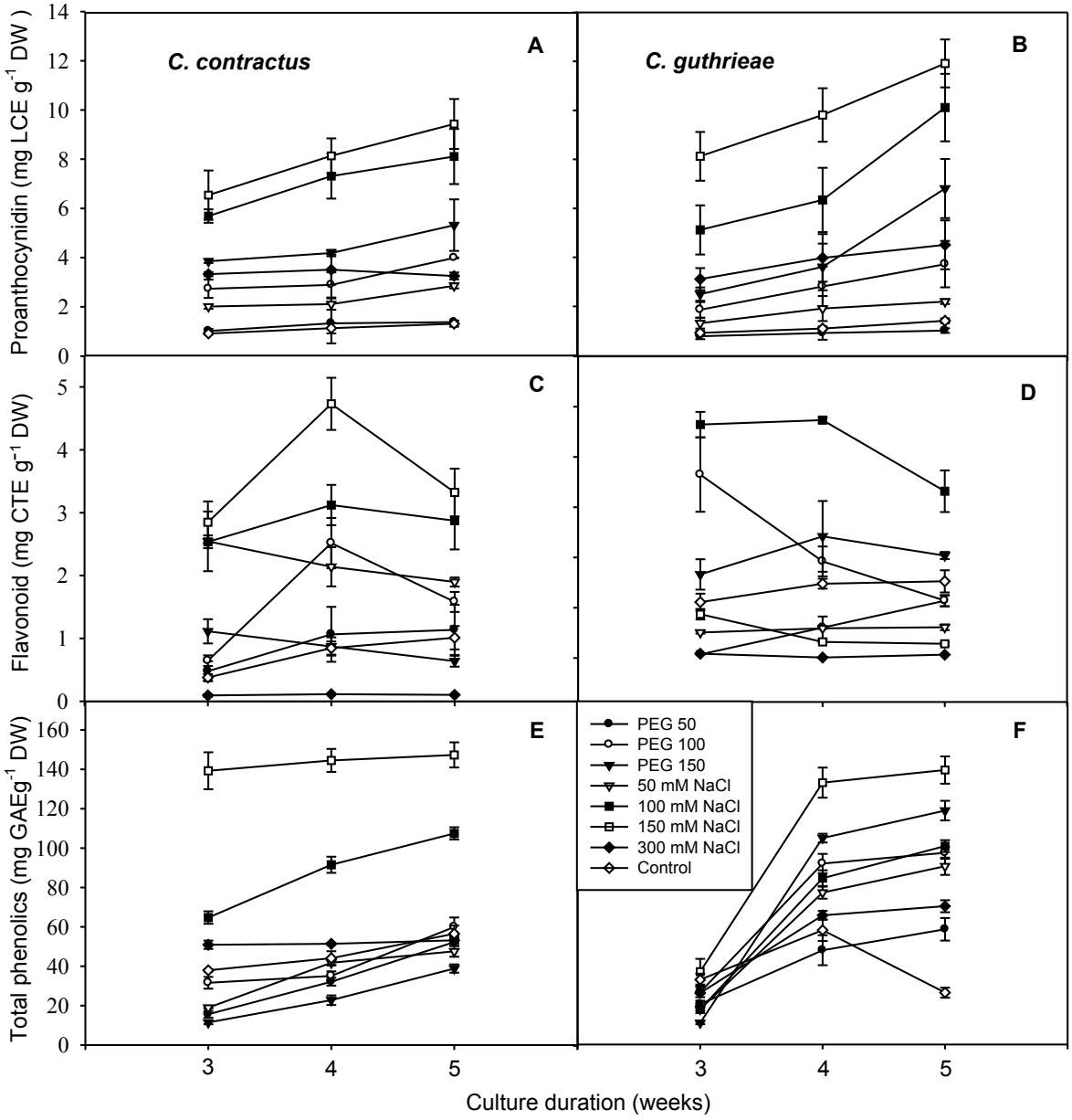


Figure 3.2. Effect of salinity and osmotic stress levels and duration on condensed tannins (proanthocyanidin) (A-B), flavonoids (C-D), total phenolic compounds (E-F) in *C. contractus* and *C. guthrieae*. Values represent means ± standard error (*n* = 3)

The most common characteristic symptom feature of an osmotically stressed plant's metabolic system is the enhanced free radical generating processes that shifts the cellular prooxidant-antioxidant equilibrium. Plants adapt to such changes in the intracellular medium to protect themselves from the oxidative damaging effects of these molecules by stimulating protective metabolic pathways. Phenolic compounds, among other secondary metabolites, form part of these complex defence

mechanisms (**SHETTY, 2004**) and thus accumulate differentially in response to the type and severity of stress. A more definite role of phenolic compounds in plant water-relations have been proposed for lipophilic resins accumulated in *Diplacus* and *Larrea* species (**RHOADES, 1977**), where an antidesiccant defence role has been assumed. Phenolic compounds, particularly flavonoids are reported to have excellent free radical-scavenging properties (**HASLAM, 1989**) and the high levels of these compounds recorded here could thus be attributed to them serving this role. In addition, the protective mechanism of flavonoids is suggested to work through antioxidant response pathways involving the biosynthesis of polymeric phenolics that lead to protective lignification of smaller polymers that act as antioxidants (**SHETTY, 2004**). Flavonoids form precursors for condensed tannins (proanthocyanidins) through spontaneous polymerisation (**HAGERMAN, 2002**). Although the two classes of compounds may be serving different roles in the defence mechanism, the sudden decline in flavonoid levels in all stress levels from week 4 to week 5 and the steady increase in the proanthocyanidin content during the same period may suggest an intermolecular conversion of the former to the later. The polymerisation of flavonoids to form condensed tannins is a spontaneous process that can be triggered by an increase in temperature (**HAGERMAN, 2002**). So under stress conditions, particularly those that dehydrate tissues, it would be logical to expect a build-up of condensed tannins and a decline in flavonoid concentrations. Under high salinity stress in this study, very low concentrations (even lower than those from the control plants) of flavonoids were recorded. This phenomenon suggests that the role of flavonoids in the amelioration of the effects of such stress levels takes less priority and that priority is perhaps diverted to other metabolites (e.g. condensed tannins in this case) that deal with the most severe stress effects. **HORNER** (**1990**) suggested a link between xylem pressure and tannin synthesis, and that the relationship can either be positive or negative, depending on the degree of water stress suffered by the plant.

In the context of the intermediary products ($NADP^+$) of proline metabolism and the positive correlation observed between proline accumulation and phenolic compounds with the increase in osmotic stress severity and duration, it is tempting to speculate a possible regulatory or metabolic cross-link between the two biosynthetic pathways. The increased $NADP^+$/NADPH ratios mediated by proline biosynthesis

has been proposed to enhance the activity of the oxidative pentose phosphate pathway and thus provide precursors to support the demand for increased secondary metabolites and nucleotide synthesis during stress (**HARE and CRESS, 1997; SHETTY, 2004**). In light of this view, the benefits of proline metabolism may be assumed to be linked more to the sensitivity of its regulatory effects on other pathways than on the accumulation of the end product itself.

One important and interesting aspect of the stress-mediated metabolic responses presented in the current study is the production of high concentrations of phenolic compounds under suboptimal growth conditions. Considering that *Cyrtanthus* species are used for medicinal purposes, with a number of medicinal properties exhibited by the phenolic compounds, makes plant stress manipulation a lucrative potential in the optimisation of phytochemicals for medicinal benefits.

3.1.3.4. Photosynthetic pigments

Optimal resource allocation and/or partitioning to balance cellular functions is key to plant survival and adaptation under suboptimal conditions. Photosynthesis drives plant metabolism by capturing photoradiation energy to synthesise carbon and nitrogen assimilates. Any parameter that curtails the smooth operation of this process would thus tilt the resource balance. Total chlorophyll (chlorophyll *a* and *b*) and carotenoid content in the stress-treated plants are presented in **Table 3.2.** Generally, no definite stress-dependent pattern in the pigment content was observed from the PEG-mediated osmotic treatments in *C. contractus*, with 100 PEG exhibiting slightly lower pigment content than 50 PEG. On the other hand, the pigments displayed a characteristic decrease with the increase in the salinity stress severity in both species. In all treatment conditions, the pigment content increased from week three to week four before dropping again in week five. In comparison with the control, the amount of pigments varied from treatment to treatment, with some exhibiting higher amounts than the control and *vice versa* in others.

Table 3.2. Effect of varying levels and duration of salinity and osmotic stress on the photosynthetic pigment content of *in vitro* regenerants of two *Cyrtanthus* species

	Treatment	Photosynthetic pigment content (µg g-1 FW)											
		Chlorophyll *a*			Chlorophyll *b*			Total chlorophyll			Carotenoid		
		Week 3	Week 4	Week 5	Week 3	Week 4	Week 5	Week 3	Week 4	Week 5	Week 3	Week 4	Week 5
C. contractus	50 PEG	249.0 ± 6.1	507.6 ± 23	186.0 ± 3.5	87.3 ± 3.1	183.7 ± 3.3	69.2 ± 1.3	336.3 ± 9.2	691.3 ± 26.3	255.2 ± 4.8	15.6 ± 0.2	26.3 ± 1.2	10.3 ± 0.3
	100 PEG	173.0 ± 3.8	382.8 ± 5.9	191.3 ± 4.1	55.7 ± 2.3	138.1 ± 1.6	71.6 ± 3.1	228.7 ± 6.1	520.9 ± 7.5	262.9 ± 7.2	13.3 ± 0.2	23.1 ± 0.8	12.1 ± 0.4
	150 PEG	248.1 ± 7.2	201.7 ± 8.8	257.8 ± 4.8	90.3 ± 5.1	121.4 ± 2.9	98.7 ± 2.3	338.4 ± 12.3	323.1 ± 11.7	356.5 ± 7.1	14.9 ± 0.3	13.9 ± 0.5	13.3 ± 0.5
	50 mM NaCl	257.2 ± 11	417.3 ± 13	306.2 ± 6.6	96.1 ± 2.4	158.0 ± 3.2	116.5 ± 2.4	353.3 ± 13.4	575.3 ± 16.2	422.7 ± 9.0	14.8 ± 0.1	25.1 ± 1.1	15.6 ± 0.7
	100 mM NaCl	200.1 ± 8.1	391.3 ± 7.5	294.5 ± 10	83.2 ± 3.8	111.1 ± 3.4	99.3 ± 2.4	283.3 ± 11.9	502.4 ± 10.9	393.8 ± 12.4	11.2 ± 01	19.7 ± 0.5	16.4 ± 0.4
	150 mM NaCl	193.2 ± 4.9	312.1 ± 5.1	271.7 ± 5.4	91.5 ±2.6	119.9 ± 1.3	102.3 ± 3.8	284.7 ± 7.5	432.0 ± 6.4	374.0 ± 9.2	11.7 ± 0.1	16.9 ± 0.7	15.9 ± 0.3
	300 mM NaCl	171.8 ± 5.9	287.9 ± 4.6	242.5 ± 5.4	88.4 ±3.2	108.3 ± 3.7	92.3 ± 2.2	260.2 ± 9.1	396.2 ± 8.3	334.8 ± 7.6	10.3 ± 0.2	15.5 ± 0.3	14.8 ± 0.2
	Control	220.1 ± 9.4	595.5 ± 9.8	243.5 ± 3.1	69.5 ± 1.9	97.8 ± 1.5	85.0 ± 3.7	289.6 ± 11.3	693.3 ± 10.3	328.6 ± 6.8	12.0 ± 0.2	16.2 ± 0.5	14.9 ± 0.4
C. guthrieae	50 PEG	142.0 ± 3.2	394.8 ± 11	291.8 ± 6.3	67.8 ± 2.1	168.0 ± 3.7	113.9 ± 3.9	209.8 ± 5.3	562.8 ± 14.7	405.7 ± 14.9	11.4 ± 0.1	27.4 ± 0.3	16.7 ± 0.4
	100 PEG	282.7 ± 7.7	353.9 ± 4.8	299.6 ± 3.3	115.9 ± 2.2	139.4 ± 3.3	133.0 ± 4.3	398.6 ± 9.9	493.2 ± 8.3	432.6 ± 7.6	21.5 ± 0.6	24.2 ± 0.4	17.3 ± 0.3
	150 PEG	260.2 ± 6.3	424.0 ± 5.2	332.4 ± 4.3	100.9 ± 2.3	156.4 ± 2.9	139.4 ± 3.3	361.1 ± 8.6	580.4 ± 8.1	471.8 ± 7.6	16.8 ± 0.1	30.8 ± 0.2	18.3 ± 0.4
	50 mM NaCl	336.5 ± 12	411.3 ± 6.9	351.6 ± 7.0	124.3 ± 2.3	173.2 ± 4.0	151.1 ± 1.9	460.8 ± 14.3	584.5 ± 10.9	502.7 ± 8.9	18.6 ± 0.2	29.3 ± 0.4	22.4 ± 0.2
	100 mM NaCl	291.0 ± 9.3	337.6 ± 4.3	314.9 ± 5.1	131.1 ± 3.4	171.7 ± 1.7	159.9 ± 2.6	422.1 ± 12.7	509.3 ± 6.0	474.8 ± 7.7	17.1 ± 0.1	25.1 ± 0.6	18.9 ± 0.2
	150 mM NaCl	229.5 ± 4.4	386.7 ±8.7	341.6 ± 2.9	120.0 ± 1.8	185.8 ± 3.5	174.2 ± 1.9	349.5 ± 6.2	572.5 ± 12.2	515.8 ± 4.8	11.5 ± 0.1	27.2 ± 0.5	19.8 ± 0.3
	300 mM NaCl	199.4 ± 3.4	283.0 ± 7.1	254.5 ± 5.7	99.2 ± 1.2	131.5 ± 2.6	106.3 ± 2.7	298.6 ± 4.6	414.5 ± 9.7	360.8 ± 8.4	10.9 ± 0.2	18.5 ± 0.4	13.7 ± 0.5
	Control	262.5 ± 6.1	534.3 ± 9.9	381.5 ± 8.3	123.1 ± 2.6	173.1 ± 4.2	150.5 ± 1.8	385.7 ± 6.7	707.4 ± 14.1	532.0 ± 10.1	20.3 ± 0.3	38.2 ± 0.2	21.4 ± 0.2
LSD ($P \leq 0.05$)													
Treatment (*C. contractus*)		12.3*	78.5*	27.8*	7.8*	11.9*	7.7*	13.1*	89..2*	16.8*	0.9*	3.2*	1.8*
Treatment (*C. guthrieae*)		19.2*	13.3*	21.7*	14.1*	11.9*	18.2*	14.8*	22.3*	10.8*	2.1*	3.7*	2.1*

Values represent mean ± standard error ($n = 3$)

*Significant at $P \leq 0.05$

Although not distinctly prominent from the results of this study, **KAPLAN et al.** (**2007**) describes water deficit-treated plants as having a higher demand than salinised plants to adjust osmotically, detoxify free radicals (reactive oxygen species), and cope with photoinhibition. Environmental factors invariably affect photosynthesis leading to imbalances between absorbed light energy and energy utilised through metabolism. The metabolic limitations of stress-induced photoinhibition to photosynthetic capacity under osmotic stress appear to primarily target photosystem II (PSII) (**BAKER, 1991; HAVAUX, 1992**). The effect of varying degrees of drought stress imposed on *Arabidopsis thaliana* showed that mild stress affects PSII functioning while moderate drought stress resulted in photosynthetic acclimation, suggesting that PSII activity does not decrease in a drought dependent pattern (**SPERDOULI and MOUSTAKAS, 2012b**). Of striking interest, in the context of a complex stress-mediated metabolic system in plants is an altered pool of metabolite composition. Reactions leading to synthesis of stress acclimation compounds become favoured and such metabolites (e.g. proline and phenolic compounds) utilise energy, carbon skeletons and nitrogen assimilates diverted from primary metabolism. This scenario presents a competition for the intermediate precursor pool between primary metabolic functions and adaptation from a limited photosynthetic capacity. Considering that the expression of chlorophyllase activity can either be enhanced or lowered, depending on the stress perception, chlorophyll content can either decrease or increase during stress (**MAJUMDAR et al., 1991; SCHURR et al., 2006**). The variations in the pigment content under different stress regimes recorded in this study and the resultant extent of photosynthetic constraint might possibly be explained on this basis. A desirable phenomenon under stress is the delayed senescence and continued accumulation of photosynthetic pigments in plants. The high proline and phenolic compound concentrations in contrast with the reduced photosynthetic pigments recorded at 300 NaCl implies perturbed plant growth as most of the assimilates from a constrained photosynthetic capacity are channelled towards adaptive metabolism. A marked reduction in growth was observed in plantlets from this treatment in comparison with those from other treatments. Carotenoids, in addition to their role in the light-harvesting function have also shown antioxidant action, protecting the photosystems (**DE PASCALE et al., 2001**). In this regard, constant levels of carotenoids would thus constitute an important stress tolerance strategy.

3.2. Carbon-nitrogen ratio and *in vitro* assimilate partitioning patterns in *Cyrtanthus guthrieae*

3.2.1. Introduction

The autotrophic nature of plant growth accounts for the efficient accumulation of carbon molecules and carbon backbones of most metabolites. Carbon fixation, accumulation and redistribution form a crucial biochemical process for structural growth, adaptation and survival of plants. In an attempt to optimise the adaptation and response to environmental cues and to integrate these with the genetically determined aspects of development, plants often shuffle and reshuffle metabolites in a survival balance between primary and secondary metabolism. Although the underlying metabolic control mechanisms remain to be elucidated in different plant species, the general metabolic complexity in plant adaptation and growth is characterised by a shift/adjustments in metabolic storage reserves, partitioning of metabolites between various sinks and an altered catabolism and synthesis of specific pools of metabolites (**WEAVER and HERMANN, 1997; LORETO and SCHNITZLER, 2010; NCUBE et al., 2012**).

Being sessile organisms, one of the most obvious challenges facing plant survival is resource availability, particularly nutrients. Nitrogen is among the crucial nutritional elements utilised as an integral component of various key physiological and intermediary metabolites in several biochemical processes. Together with carbon (C) assimilates, the relative quantity of nitrogen (N) molecule reserves and/or demands, are thought to have a profound influence on the partitioning patterns of the various assimilates of specific metabolites (**BRYANT et al., 1983; HERMS and MATTSON, 1992**). A coordinated mechanism of C and N metabolism is thus necessary since N assimilation is energetically costly and requires C skeletons in the form of 2-oxoglutarate, ATP, and reductants (**OLIVEIRA and CORUZZI, 1999; LANCIEN et al., 2000**) Fundamental to this metabolic balance, is the consequent regulatory effects of certain metabolites such as sucrose and amino acids on the enzymes involved in nitrogen assimilation and hence their importance to whole plant

physiology (**SHEEN, 1990**). In this sense, the availability of precursor molecules and products represents a fundamental input for the control of gene expression and enzyme activities to allow coordination of C and N assimilation in the light of growth and developmental demands. Inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, asparagine, and aspartate, which then serve as important nitrogen carriers in plants (**LAM et al., 1996**). The assimilation of C and N nutrients is tightly linked to almost every biochemical pathway (**STITT and KRAPP, 1999**). Their role in stimulating plant growth is not only limited to metabolic effects, but also to the fact that plants modulate their growth according to the perception of the resources available in the growing medium. For effective and efficient utilisation of the often limited nitrogen assimilates, plants have evolved mechanisms to recycle nitrogen released during various catabolic reactions.

In this context, it is logical to assume that regulatory systems of C and N metabolism are essential for the maintenance of a physiologically relevant C:N ratio for constant plant growth in response to different environmental regimes. A tilt in this balance away from a steady state situation that allows constant growth and development, by either N or C resource deprivation, leads to high demands for either of the two assimilates. To adapt to the new environmental or physiologically imposed equilibrium, plants adjust their metabolism by prioritising or up-regulating specific biochemical pathways that lead to accumulation/consumption of preferred metabolites for sustained survival under resource limitations. Apart from products of primary metabolism, secondary products also contribute to the metabolic pool of compounds that are affected by plant resource dynamics and adaptation (**NCUBE et al., 2012**). In particular, source-sink relations are expected to constrain the levels of secondary metabolites accumulated by growing plants (**PRICE et al., 1989**) but their dynamics are presumably characteristics of each species under a given set of physiological and/or environmental regimes.

In light of the fact that the therapeutic value of medicinal plants is derived from an accumulated pool of secondary metabolites, this study was conducted as an *in vitro* manipulation of the nutrient resources in the culture medium to determine the partitioning patterns of assimilates to various metabolites in *Cyrtanthus guthrieae*. The underlying principle, central to the understanding of plant metabolism is the

detection and quantification of metabolic fluxes (**STEPHANOPOULOS, 1999**). Increased quantitative knowledge on plant metabolism provides a basis for opportunities towards manipulation/alterations of either plants and/or their environment to optimise production. An in-depth analysis of compositional changes of callus-derived *in vitro C. guthrieae* regenerants in response to varying levels of C and N in the culture medium is described in this study, with the objective of determining the possible metabolic routes within the species' metabolism that carry significant fluxes and the resultant levels of these metabolites.

3.2.2. Materials and methods

3.2.2.1. Plant material and growth conditions

Callus-derived *in vitro*-regenerated *C. guthrieae* plantlets (**Figure 2.4**, Chapter 2) obtained though the indirect shoot organogenesis procedure described in **Section 2.2.5.2** were used for this experiment. The unrooted plantlets were multiplied on optimised shoot proliferation MS medium (**Section 2.2.2**) for 8 weeks before sub-culturing in different (C-N) media treatments at 25 ± 2 °C with a 16 h photoperiod. Unless specified otherwise, all culture environmental conditions were as described in **Section 2.2.2**.

3.2.2.2. C and N nutrient manipulation treatments

Nitrogen (N) and carbon (C) levels were achieved by manipulating the concentration levels of the different nitrogen sources (NH_4NO_3 and KNO_3) in MS culture medium supplemented with different sucrose (carbon) levels. Proliferative plantlet cultures were separated into uniformly sized shoot explants and placed into screw cap culture jars containing 30 ml MS medium with different C and N concentrations (**Table 3.3**). The cultures were maintained in growth conditions as described in **Section 2.2.2**. Each treatment with 30 plantlets was repeated twice and the data from the two replicates were combined for data analysis. The cultures were harvested after 6 weeks of culture and samples from each treatment were washed, freeze-dried immediately in liquid nitrogen and stored at -70 ºC before being used for various biochemical analyses.

3.2.2.3. Relative growth rate

The relative growth rates (RGR) of cultured plantlets from each treatment were calculated at harvest. The initial dry weight (DW) of the plantlet cultures were determined by randomly taking two replicates of similar size and number of explants representative of those used in treatment cultures at the same time during initiation of treatments. The two replicate samples were oven-dried to a constant weight at 70 °C and the following formula was used to calculate the relative growth rates:

$$RGR\ (d^{-1}) = [(X_{Dn} - X_{D0})/t]/\ X_{D0}$$

where, X_{Dn} is the sample DW at harvest, X_{D0} is the initial sample DW and t is the duration of culture in days.

3.2.2.4. Determination of free amino acids

Extraction and estimation of free amino acids was done following the method of **MOORE and STEIN** (**1948**) with slight modifications. Plant samples (100 mg) were homogenised in a pestle and mortar with 10 ml of boiling 80% (v/v) aqueous ethanol. The extracts were centrifuged at 1 000 *g* for 15 min and the supernatants made up to 10 ml with 80% ethanol and used for the estimation of free amino acids. Into test tubes, 1 ml of each ethanol extract was transferred and neutralised with 0.1N sodium hydroxide, to which, 1 ml ninhydrin reagent [8 g in 100 ml 2-methoxyethanol with 0.2 M citrate buffer, pH 5.0 (100 ml)] was added. The reaction mixtures were incubated in a boiling water bath for 20 min before 5 ml of diluting reagent [50% (v/v) aqueous *n*-propanol] were added and cooled in a cold water bath. The absorbance was read at 570 nm using UV-vis spectrophotometer (Varian Cary 50, Australia). A leucine (Sigma-Aldrich) standard curve was prepared from a freshly made stock solution (0.5 mg ml^{-1}) in distilled water. The amino acid contents were calculated from the standard curve prepared and expressed in μmol g^{-1} DW.

3.2.2.5. Protein quantification

Proteins were extracted from the freeze-dried plant tissue samples (50 mg) thrice in 1 ml phosphate buffer (0.2 M, pH 7.2) containing 0.014 M β-mercaptoethanol by homogenisation at 4 °C for 20 min. The extracts were centrifuged for 15 min at 8 000 *g* at 4 °C and the supernatants from the three extractions pooled together. Protein content was measured following the **BRADFORD** (**1976**) method. In duplicate, 0.5 ml of each filtrate sample were transferred into test tubes into which 5 ml of protein reagent were added to each tube and mixed well by gentle vortex mixing. Protein reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G250 (Sigma-Aldrich) in 50 ml of 95% (v/v) aqueous ethanol. The solution was then mixed with 100 ml of 85% (v/v) phosphoric acid and made up to 1000 ml with distilled water before being filtered in Whatman No. 1 filter paper and stored in a dark bottle. Absorbance was read at 595 nm using a UV-vis spectrophotometer against a blank that contained extraction buffer instead of sample. Freshly prepared bovine serum albumin (BSA) (1 mg^{-1} ml) in distilled water was used to determine the standard curve and protein content expressed in mg g^{-1} DW.

3.2.2.6. Determination of soluble sugars and starch

For each treatment sample, 50 mg of the freeze-dried plant material (stored at -70 ºC) were extracted three times with 5 ml of 80% (v/v) aqueous ethanol, by boiling the samples in glass tubes capped with glass marbles in a water bath at 95 °C for 10 min. After extraction, the tubes were centrifuged at 3 000 *g* for 5 min, and the supernatants for the three extractions combined. The sugar concentrations were determined using the phenol-sulphuric acid method (**DUBOIS et al., 1956**) with slight modifications. In test tubes, 1 ml aliquots of sugar extracts were mixed with 0.5 ml of 5% (v/v) aqueous phenol and 2.5 ml of concentrated sulphuric acid. The test tubes containing the reaction mixtures were allowed to stand for 10 min, vortexed for 30 s before being placed in a water bath for 20 min at room temperature to allow colour development. Absorbance was then read at 490 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia) against a blank prepared in a similar way but with 1 ml distilled and deionised (DDI) water in place of sugar extracts.

Glucose (0.1 mg^{-1} ml) was used as the standard and soluble sugars were expressed in glucose equivalents (GE) (mg GE g^{-1} DW).

Starch was extracted from the ethanol residues (from soluble sugar extractions) as described by **SMITH and ZEEMAN** (**2006**) and quantified similarly as described above for sugars, with glucose as a standard. After allowing ethanol to completely evaporate from the extract residues, 1 ml of distilled water was added and homogenised to a smooth consistency. The volume of the homogenates were made up to 5 ml with distilled water washings from the mortar. In test tubes, the homogenates were tightly caped and heated at 100 ºC for 10 min to gelatinise starch granules. After cooling at room temperature, 0.5 ml of each homogenate sample (in triplicates) was transferred into new test tubes and 0.5 ml sodium acetate buffer (0.2 M, pH 5.5) added. To two of the test tubes for each sample, approximately 6 units of the enzyme α-amyloglucosidase (EC 3.2.1.3) (Sigma-Aldrich) and at least 0.5 units of α-amylase (EC 3.2.1.1) (Sigma-Aldrich) were added. The third test tube without the enzyme solution for each sample served as the control. The freshly prepared enzyme solution contained 0.1 M 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 7.5, 0.5 mM ATP, 1 M NAD, and 4 mM $MgCl_2$. The reaction mixtures were then centrifuged at 10 000 *g* for 5 min at room temperature to remove particulate material. The samples of the supernatants were assayed for starch similarly as described above for soluble sugars, with glucose as a standard (mg GE g^{-1} DW).

3.2.2.7. Alkaloid content

Dried plant tissue samples (1 g) were extracted with 20 ml of ethanol in a sonication bath with ice for 2 h. The extracts were then filtered under vacuum through Whatman No. 1 filter paper and concentrated *in vacuo* at 35 °C using a rotary evaporator. The obtained residues were each dissolved in 2N HCl (2 ml) from which 1 ml was washed 3 times with 10 ml chloroform in a separation funnel. The resulting extract solution was adjusted to neutral pH with 0.1N NaOH before adding 5 ml bromocresol green (BCG) (BDH chemicals, England) (0.1 M) and 5 ml phosphate-citrate buffer (0.8 M, pH 4.7). After vigorous shaking, the complex mixtures were extracted 3 times with a total volume of 10 ml chloroform. Absorbance was then read at 470 nm

(**SHAMSA et al., 2008**) using a UV-vis spectrophotometer and total alkaloids calculated from an atropine standard curve and expressed as µg atropine equivalents (AE) per g DW.

3.2.2.8. Polyphenols and proline quantification

Polyphenolic content were determined from dried plant samples as described earlier; total polyphenol content (**Section 3.1.2.5**), flavonoids (**Section 3.1.2.6**), and proanthocyanidins (**Section 3.1.2.7**). Proline contents were quantified as described in **Section 3.1.2.3**.

3.2.2.9. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS software for Windows (IBM SPSS®, version 21.0, Chicago, IL, USA). Where there were significant differences ($P \leq 0.05$), the means were further separated using Duncan's multiple range test (DMRT) and/or Least Significant Difference (LSD).

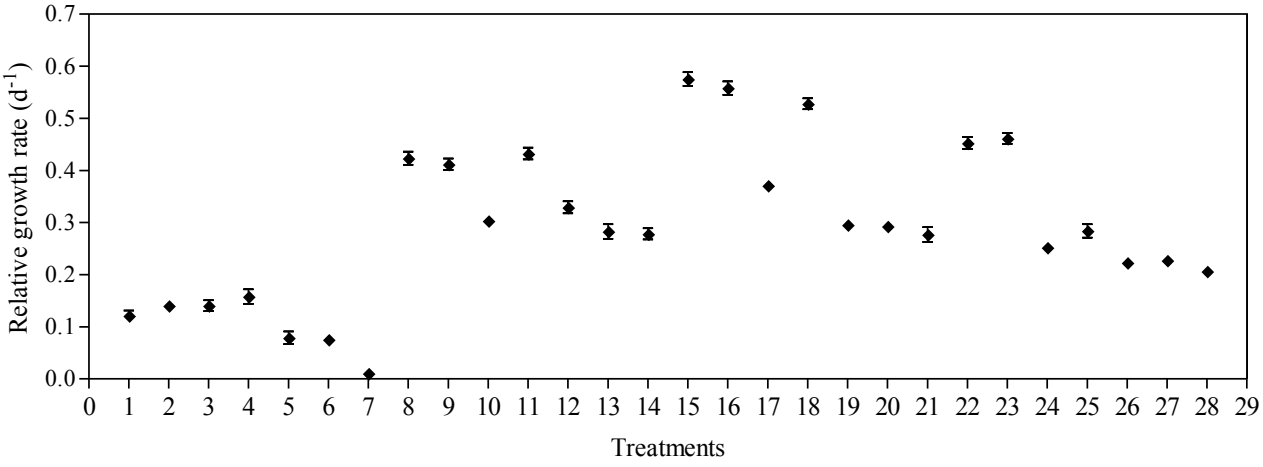
Table 3.3. Carbon and nitrogen concentrations and combinations of the MS media used as treatments in this study

Carbon source (C)	Nitrogen source (N)	
Sucrose (mM)	NH_4NO_3(mM)	KNO_3 (mM
3	41.2	37.6
	20.6	18.8
	5.3	4.7
	10.3	-
	5.3	-
	-	9.4
	-	4.7
15	41.2	37.6
	20.6	18.8
	5.3	4.7
	10.3	-
	5.3	-
	-	9.4
	-	4.7
88	41.2	37.6
	20.6	18.8
	5.3	4.7
	10.3	-
	5.3	-
	-	9.4
	-	4.7
175	41.2	37.6
	20.6	18.8
	5.3	4.7
	10.3	-
	5.3	-
	-	9.4
	-	4.7

3.2.3. Results and discussion

3.2.3.1. Relative growth rate

The relative growth rates of *in vitro* callus-derived *C. guthrieae* plantlets cultured on media with different carbon and nitrogen concentrations are shown in **Figure 3.3**. The highest relative growth rate (0.58 ± 0.013 d^{-1}) was obtained at a medium combination of 88 mM sucrose, 41.2 mM NH_4NO_3 and 37.6 mM KNO_3 although not significantly different but slightly higher than that from 88 mM sucrose, 20.6 mM NH_4NO_3 and 18.8 mM KNO_3 (0.56 ± 0.013 d^{-1}). The lowest RGR was recorded at a medium with limited C and N concentrations (3 mM sucrose and 4.7 mM KNO_3). Generally, RGRs increased proportionally with an increase in C concentrations in the medium up to the 88 mM sucrose level but did not seem influenced by any further increase beyond this level. Correspondingly, the high N concentrations in each of the C levels in the medium resulted in the high RGR in each of the 4 different C concentration regimes. Consistent with these findings, is the fact that dry matter accumulation demands an enormous investment in C skeletons particularly in the form of structural carbohydrates. However, the decrease in growth rate under N-limited medium regimes, irrespective of the sufficiently high C supplies in the media, is thought to be partly attributed to metabolic limitations and the fact that it serves as an important adaptive response to prevent endogenous N depletion (**STITT and KRAPP, 1999; RAHAYU et al., 2005**). Nitrate-derived signalling mechanisms regulate N- and influence C metabolism and consequently adjust growth and an array of other physiological processes in plants (**HOFF et al., 1994; SCHEIBLE et al., 1997; SMEEKENS et al., 2010**). In this regard, not only do the proportions of C and N in the growing medium influence plant growth, but to a greater extent dictate the resource partitioning patterns to various other sinks. The extent of this signalling influence and the complexity of the resulting sinks and pools of metabolites generated, however, vary with species.



1. 3 mM C, 41.2 mM NH 37.6 KN; **2.** 3 mM C, 20.6 mM NH 18.8 mM KN; **3.** 3 mM C, 5.3 mM NH 4.7 mM KN; **4.** 3 mM C, 10.3 mM NH; **5.** 3 mM C, 5.3 mM NH; **6.** 3 mM C, 9.4 mM KN; **7.** 3 mM C, 4.7 mM KN; **8.** 15 mM C, 41.2 mM NH 37.6 KN; **9.** 15 mM C, 20.6 mM NH 18.8 mM KN; **10.** 15 mM C, 5.3 mM NH 4.7 mM KN; **11.** 15 mM C, 10.3 mM NH; **12.** 15 mM C, 5.3 mM NH; **13.** 15 mM C, 9.4 mM KN; **14.** 15 mM C, 4.7 mM KN; **15.** 88 mM C, 41.2 mM NH 37.6 KN; **16.** 88 mM C, 20.6 mM NH 18.8 mM KN; **17.** 88 mM C, 5.3 mM NH 4.7 mM KN; **18.** 88 mM C, 10.3 mM NH; **19.** 88 mM C, 5.3 mM NH; **20.** 88 mM C, 9.4 mM KN; **21.** 88 mM C, 4.7 mM KN; **22.** 175 mM C, 41.2 mM NH 37.6 KN; **23.** 175 mM C, 20.6 mM NH 18.8 mM KN; **24.** 175 mM C, 5.3 mM NH 4.7 mM KN; **25.** 175 mM C, 10.3 mM NH; **26.** 175 mM C, 5.3 mM NH; **27.** 175 mM C, 9.4 mM KN; **28.** 17 mM C, 4.7 mM KN (C = sucrose; NH = NH_4NO_3; KN = KNO_3)

Figure 3.3. Relative growth rates (RGR) of *in vitro*-cultured *C. guthrieae* plantlets in response to varying levels and combinations of carbon and nitrogen in the culture media. Bars represent mean ± standard error (*n* = 3)

Signals derived from internal and external nitrate, sucrose and phosphate also adjust root growth and architecture to the physiological state of the plant, and the distribution of nitrate in the medium (**JAIN et al., 2007**). In addition, changes in the sucrose supply lead to coordinated reprogramming of carbon and nitrogen metabolism. For example, sugar depletion leads to a collapse of the nitrate reductase transcript and protein, post-translational inactivation of nitrate reductase and an inhibition of nitrate assimilation (**MATT et al., 2002**), the induction of many genes that are involved in the catabolism of amino acids, nucleotides and other cellular components and an inhibition of growth (**GIBON et al., 2004**). Such multidimensional regulation and signalling mechanisms could have possibly interacted to tilt growth in the direction observed in this study.

A closer comparison of *C. guthrieae* growth rates under two media extremes; the C-limited with high/low N concentration media and the ones with excess C and N supplies, indicates the existence of an optimal C:N ratio for constant optimal growth. Under C-limited regimes, growth is constrained by excess N and picks up as N concentration reduces but drops sharply again as N becomes limiting. On the other hand, when both of these macronutrients are in excess for optimum growth (175 mM sucrose, 41.2 mM NH_4NO_3 and 37.6 mM KNO_3) in the growth media, RGR is reduced to levels significantly lower than the optimum recorded in this study but remains higher than those under conditions of C limitation. In a complete functional autotrophic system, a decrease in the photosynthetic rate severely impairs N metabolism and *vice versa* when N availability is limited in the growing environment (**LONGSTRETH and NOBEL, 1980; KROUK et al., 2010**), a phenomenon that emphasises the interdependence between C assimilates and N metabolism in modulating plant growth and development. One strikingly interesting characteristic trend observed in this study, is the favourable and preferential growth rates recorded at all C concentrations supplemented with low amounts of N in the form of NH_4NO_3 (10.3 and 5.3 mM) in contrast with KNO_3 (9.4 and 4.7 mM). This characteristic response of plant growth to different N forms, points to yet another dimension of the C-N ratio, in that the N source appears to play a significant role. One possible explanation for this attribute could perhaps lie in the fact that all inorganic nitrogen is first reduced to ammonia (NH_4^+) before it is incorporated into organic forms (**HOFF et al., 1994**) and hence NH_4NO_3 would thus be preferred and more readily utilised. Compared to the nitrate ion (NO_3^-), the ammonium ion (NH_4^+) is the most highly 'reduced' form and the reduction of NO_3^- to NH_4^+ is an energetically costly process that demands considerable expenditure of fixed C in addition to it being taken up against a steep electrochemical gradient in a slightly acidic culture medium (pH 5.8). To more effectively utilise resources without severe growth penalties in C-limited regimes, medium with NH_4^+ as the N source would likely be expected to yield favourable growth rates. The ammonium ion is rapidly absorbed into the root cells even in the presence of NO_3^- leading to its pronounced accumulation (**BRITTO and KRONZUCKER, 2013**) and possibly subsequent utilisation and growth. However, deficiency, sufficiency and toxicity occur for both inorganic N forms, with very different set points, and growth optima are expected and commonly observed (**CABRERA, 2000; GAN et al., 2012**). **BRITTO and KRONZUCKER** (**2013**) suggest

that such optimum curves, and how they differ with N source for a given plant species, should inform any fundamental appraisal of the N-source preference(s) of that species, since they are directly linked with plant productivity.

3.2.3.2. Patterns of primary metabolites accumulation

The levels of primary metabolites varied markedly with the varying levels and combinations of C and N in the culture media (**Table 3.4**). Soluble organic solutes like sugars and amino acids serve as precursor molecules as well as energy sources to a number of biochemical processes in plants. At all the tested C and N concentration levels in this study, the endogenous pools of soluble sugars in *C. guthrieae* tissue cells ranged between 2.83 and 9.33 mg g^{-1} DW. The media with low C concentrations had the least amount of soluble sugars with correspondingly high levels recorded from the medium with high C concentration. Within each C concentration category, regardless of the form and concentrations of N supplements, there seemed to be no significant differences in the total soluble sugars accumulated although significant variations were recorded between categories. This type of accumulation pattern points to a clear lack of and/or minor influential role played by N in determining the pools of these metabolites but to be more under the influence of C availability. Although theoretical logic would partly attest to this conclusion, the fact that none of the treatments completely lacked N in this study, might probably mean that the amount of N furnished in each of the treatments did not amount to a metabolic limitation of sugar biosynthesis. The insignificant differences in the amount of endogenous sugar content from culture samples cultivated on 15 mM and 88 mM sucrose suggest that C limitation on the biosynthesis of these metabolites only proceeds to this level and that it is the optimum for the species under these nutrient levels. An increase in sucrose and inorganic salt concentrations in the culture medium exerts some osmotic properties leading to the progressive reduction in water potential. For example, 175 mM media sucrose concentration in this study amounts to -0.461 MPa of water potential at 25 ºC, an amount of which constitutes an osmotic strain to the growing plant cells. As a reflection of this moisture constraint, plantlets cultured under these treatment conditions in this study adjusted their metabolism to invest extra resources to alter their pool of metabolites in favour of soluble organic osmolytes like sugars, amino acids and proline (**Table 3.4**). Plant

cells maintained in an environment with low osmotic potential suffer from water loss and consequently this sets in motion changes in metabolism with the resultant accumulation of endogenous osmolytes to ensure continuous moisture absorption and pressure potential sufficient for sustained growth (**VERSLUES et al., 2006; BRITTO and KRONZUCKER, 2013**). The properties of proline, among other osmolytes, allows for its preferential accumulation by most plant species to adjust cellular osmolarity. Despite the fact that proline demands N investment into its structure, significant amounts of this compound were accumulated in tissues of plantlets cultured in low osmotic potential medium (175 mM sucrose) with limited N supplements. The metabolic commitment of the limited N resources by *C. guthrieae* to proline under osmotic stress, ahead of other N-requiring soluble osmolytes like amino acids, asserts to its purported adaptive role under stress regimes (**VERSLUES et al., 2006; HARE and CRESS, 1997**). Osmotic stress perception and signalling in plants, is translated into biochemical reactions, metabolic and physiological adjustments, with consequent changes in the progression of growth and development (**BOHNERT and SHEVELEVA, 1998**). Coupled with the role of nutrients in stimulating plant growth, plants modulate their growth according to the perception of the resources available in the growing environment. In the present study, plant growth was perturbed under conditions of high sucrose concentration (175 mM) even with sufficient quantities of N and progressively declined as N became limiting (**Figure 3.3**). Conversely to this observed growth pattern, the biosynthesis of N-containing soluble metabolites like amino acids and proline became favoured, an indication of trade-off in assimilate partitioning between growth and adaptation.

Table 3.4. Effect of varying concentrations and combinations of carbon and nitrogen nutrients in the culture media on the *in vitro* accumulation patterns of primary metabolites in *C. guthrieae*

Treatment (mM)			Organic compounds				
			Total amino acids	Total protein	Proline	Soluble sugars	Starch content
Sucrose	NH_4NO_3	KNO_3	(µmol g^{-1} DW)	(mg g^{-1} DW)	(µmol g^{-1} FW)	(mg GE g^{-1} DW)	(mg GE g^{-1} DW)
3	41.2	37.6	15.4 ± 0.32	1.71 ± 0.01	0.81 ± 0.01	3.31 ± 0.14	0.3 ± 0.01
	20.6	18.8	17.1 ± 1.03	1.53 ± 0.05	0.79 ± 0.01	3.14 ± 0.18	0.4 ± 0.01
	5.3	4.7	1.4 ± 0.03	1.33 ± 0.02	0.77 ± 0.02	3.26 ± 0.17	0.3 ± 0.02
	10.3	-	6.4 ± 0.78	1.31 ± 0.01	0.78 ± 0.03	3.21 ± 0.18	0.0 ± 0.00
	5.3	-	3.4 ±0.05	1.22 ± 0.08	0.80 ± 0.07	2.91 ± 0.10	0.1 ± 0.04
	-	9.4	1.4 ± 0.07	1.21 ± 0.04	0.86 ± 0.05	2.83 ± 0.13	0.2 ± 0.00
	-	4.7	0.7 ± 0.02	1.11 ± 0.07	0.79 ± 0.04	2.88 ± 0.12	0.2 ± 0.03
15	41.2	37.6	22.2 ± 0.14	2.13 ± 0.06	0.83 ± 0.09	5.66 ± 0.21	1.3 ± 0.08
	20.6	18.8	19.8 ± 0.72	2.30 0± 0.11	0.87 ± 0.06	5.89 ± 0.19	1.2 ± 0.09
	5.3	4.7	14.2 ± 0.92	1.94 ± 0.08	0.81 ± 0.06	5.93 ± 0.16	1.6 ± 0.10
	10.3	-	13.1 ± 0.77	1.52 ± 0.00	0.76 ± 0.02	5.87 ± 0.16	1.6 ± 0.11
	5.3	-	7.3 ± 0.53	1.23 ± 0.01	0.80 ± 0.01	5.61 ± 0.12	1.0 ± 0.05
	-	9.4	2.1 ± 0.11	1.91 ± 0.07	0.83 ± 0.01	5.81 ± 0.20	1.5 ± 0.12
	-	4.7	1.9 ± 0.08	1.31 ± 0.03	0.87 ± 0.04	5.91 ± 0.15	1.5 ± 0.07
88	41.2	37.6	29.1 ± 0.31	2.31 ± 0.08	1.21 ± 0.03	6.11 ± 0.21	12.1 ± 0.31
	20.6	18.8	28.7 ± 0.43	2.28 ± 0.11	1.27 ± 0.13	6.43 ± 0.13	11.3 ± 0.23
	5.3	4.7	19.1 ± 0.88	2.04 ± 0.25	1.01 ± 0.10	6.27 ± 0.18	10.8 ± 0.17
	10.3	-	17.3 ± 1.01	2.10 ± 0.07	0.93 ± 0.07	6.27 ± 0.21	10.5 ± 0.15
	5.3	-	13.8 ± 0.64	1.61 ± 0.02	0.84 ± 0.04	6.33 ± 0.15	10.4 ± 0.21
	-	9.4	1.3 ± 0.66	1.89 ± 0.05	0.78 ± 0.05	6.38 ± 0.11	10.7 ± 0.20
	-	4.7	0.8 ± 0.51	1.65 ± 0.08	0.81 ± 0.10	6.35 ± 0.17	10.8 ± 0.14
175	41.2	37.6	42.2 ± 1.02	1.95 ± 0.06	2.23 ± 0.17	8.71 ± 0.28	8.3 ± 0.11
	20.6	18.8	38.1 ± 0.98	1.87 ± 0.04	2.21 ± 0.21	8.45 ± 0.19	8.6 ± 0.21
	5.3	4.7	15.3 ± 0.27	1.81 ± 0.09	2.23 ± 0.14	8.95 ± 0.23	10.1 ± 0.16
	10.3	-	18.1 ± 0.61	1.74 ± 0.08	1.82 ± 0.13	9.11 ± 0.18	8.6 ± 0.17
	5.3	-	4.4 ± 0.16	1.78 ± 0.07	1.33 ± 0.11	9.33 ± 0.22	9.4 ± 0.27
	-	9.4	4.1 ± 0.32	1.69 ± 0.05	1.73 ± 0.18	8.99 ± 0.19	9.5 ± 0.24
	-	4.7	2.8 ± 0.37	1.69 ± 0.09	1.48 ± 0.09	9.14 ± 0.17	8.9 ± 0.19
LSD ($P \leq 0.05$)			1.87*	0.63*	0.16*	1.03*	1.53*

Mean values ± standard error (n = 3).
*Significant at $P \leq 0.05$

Starch deposition in plant tissues increased as the amount of C increased in the medium (**Table 3.4**), with the highest concentration (12.1 mg g^{-1} DW) recorded from plantlets cultivated under 88 mM sucrose, 41.2 mM NH_4NO_3 and 37.6 mM KNO_3. In the extreme C-limited treatment regime (3 mM sucrose) starch deposition in plant tissues was minimal and insignificant, but reached its peak when the sucrose level was increased to 88 mM before dropping slightly when the sucrose level doubled to 175 mM. This therefore, suggests that part of the excess carbon apart from that utilised for growth, adaptive acclimation and maintenance was converted to starch and stored in plant tissues. Although protein synthesis appeared to follow a somewhat characteristic concentration increase with an increase in C levels in the medium, this trend was apparent in the upper ranges of N concentrations. In this context, it is conceivable to assume that protein synthesis was not a competitive sink for starch in terms of carbon resources and that accumulation of protein is mostly influenced by N availability. The very minor significant differences in protein accumulation among the different treatments in this study could mean that most of the protein may be present constitutively in plant tissues and is only slightly altered to adjust for the differences in media conditions. Cells preferentially synthesise specific proteins either as structural proteins or mostly enzymes in an adaptive response to cope in stress conditions (**MANSOUR, 2000**). The high levels of protein content detected from the medium with low osmotic potential could thus be partly explained on this basis. Proteins that are accumulated in plant tissues subjected to osmotic stress or conditions of excess N could possibly be involved in osmotic adjustments or serve a storage role for N for remobilisation after stress relief and/or in situations of N starvation (**RABE, 1990; SINGH et al., 1987**). The quantitatively elevated levels of free amino acids and soluble sugars under conditions of high sucrose concentration (175 mM) in the medium at a metabolic cost of reduction in starch content and RGR may suggest that cells primarily respond to water stress by altering assimilation of these metabolites with possible remobilisation from polymer structures. This, therefore, would entail that the processes that conserve or generate these osmolytes limit the biosynthesis of polymer molecules such as starch and thus constrain growth.

3.2.3.3. Secondary metabolites

The strong imbalance in the nutrient medium between C and N presented by some treatments in the low and high sucrose concentrations in the present study, and the close interactions in the metabolism of the two macronutrients, yielded some interesting levels of secondary metabolites (**Table 3.5**). The levels of polyphenols increased progressively with a C concentration increase in the culture medium. Generally, total phenolic contents in particular were significantly higher in cases where N was limiting. Counterintuitively as this may seem considering the fact that *C. guthrieae* plantlets growing in these treatment media pay a growth penalty (**Figure 3.3**), the results could be somehow consistent with the carbon/nitrogen balance (CNB) hypothesis (**BRYANT et al., 1983**). The theory rests on the assumptions that plants prioritise resources primarily to growth and that C and N are allocated to secondary metabolite production only after the requirements for growth are met. It further assumes that availability of precursor molecules would thus determine the rate of secondary metabolite synthesis (**REICHARDT et al., 1991**). In line with this school of thought, in conditions where N limits growth coupled with a saturated pool of C skeletons (carbohydrates), synthesis of carbon-based secondary metabolites like polyphenols would, logically be favoured. Similarly, the theory predicts that N limitation in the nutrient medium leads to a decrease in the production of N-containing compounds such as alkaloids and cyanogenic glycosides. The results from this study seem to be somehow consistent with this logic. Total polyphenolic, proanthocyanidin and flavonoid contents were higher at 175 mM sucrose with 10.3 and 5.3 mM NH_4NO_3 respectively. In contrast, at 88 mM sucrose concentration, the highest total polyphenols and flavonoids were obtained when the source of N was KNO_3 (9.4 and 4.7 mM) as opposed to NH_4NO_3 at 175 mM sucrose level. This suggest that N deficiency perception and metabolic response in *C. guthrieae* is a subject of both C:N ratio and the form of N available in the medium.

Table 3.5. Effect of varying concentrations and combinations of carbon and nitrogen nutrients in the culture media on the *in vitro* accumulation patterns of alkaloids and polyphenols in *C. guthrieae*

Treatment (mM)			Secondary metabolites			
			Total alkaloids	Total phenolic content	Flavonoid content	Proanthocyanidin
Sucrose	NH_4NO_3	KNO_3	(µg AE g^{-1} DW)	(mg GAE g^{-1} DW)	(mg CTE g^{-1} DW)	(µg LCE g^{-1} DW)
3	41.2	37.6	283.2 ± 1.73	9.3 ± 0.33	0.89 ± 0.05	312.4 ± 6.76
	20.6	18.8	214.0 ± 4.51	9.1 ± 0.12	0.92 ± 0.08	327.1 ± 5.13
	5.3	4.7	146.8 ± 3.47	7.1 ± 0.26	0.69 ± 0.10	232.4 ± 3.16
	10.3	-	181.7 ± 4.17	7.3 ± 0.74	1.01 ± 0.23	187.9 ± 3.73
	5.3	-	127.3 ± 5.44	5.8 ± 1.21	0.63 ± 0.18	167.4 ± 2.81
	-	9.4	112.1 ± 1.77	6.3 ± 0.44	0.71 ± 0.13	154.5 ± 6.17
	-	4.7	101.9 ± 2.91	5.1 ± 0.51	0.94 ± 0.21	157.2 ± 7.54
15	41.2	37.6	312.1 ± 3.62	23.4 ± 1.37	1.21 ± 0.63	217.2 ± 4.32
	20.6	18.8	273.5 ± 1.23	27.1 ± 1.58	1.38 ± 0.51	147.0 ± 5.17
	5.3	4.7	127.8 ± 2.91	25.7 ± 2.01	1.52 ± 0.55	228.9 ± 3.55
	10.3	-	146.8 ± 1.56	32.3 ± 1.08	1.61 ± 0.73	171.8 ± 2.10
	5.3	-	121.3 ± 4.81	20.4 ± 1.18	1.46 ± 0.47	163.3 ± 5.71
	-	9.4	123.4 ± 2.01	23.4 ± 1.97	1.38 ± 0.11	157.9 ± 3.88
	-	4.7	98.6 ± 4.23	27.7 ± 2.11	1.51 ± 0.71	141.8 ± 5.66
88	41.2	37.6	97.2 ± 3.71	48.3 ± 2.33	1.88 ± 0.08	466.5 ± 7.10
	20.6	18.8	109.4 ± 2.66	41.1 ± 1.84	1.72 ± 0.13	351.7 ± 5.62
	5.3	4.7	90.2 ± 1.52	51.6 ± 1.72	1.94 ± 0.17	483.1 ± 6.66
	10.3	-	103.0 ± 2.55	69.4 ± 2.86	1.11 ± 0.21	416.3 ± 3.91
	5.3	-	111.3 ± 1.38	77. 3 ± 3.01	1.48 ± 0.19	320.4 ± 4.78
	-	9.4	107.4 ± 1.88	86.1 ± 1.03	2.01 ± 0.22	318.6 ± 5.84
	-	4.7	92.7 ± 2.46	82.3 ± 3.47	2.71 ± 0.21	289.5 ± 2.94
175	41.2	37.6	268.4 ± 2.22	63.2 ± 3.35	3.18 ± 1.01	472.3 ± 5.42
	20.6	18.8	216.4 ± 1.30	85.7 ± 2.77	3.34 ± 0.98	566.4 ± 4.86
	5.3	4.7	95.42 ± 1.80	88.4 ± 3.75	4.11 ± 1.11	676.9 ± 5.45
	10.3	-	98.8 ± 2.65	101.0 ± 1.88	4.88 ± 0.92	711.2 ± 8.31
	5.3	-	83.4 ± 3.89	95.5 ± 2.61	4.51 ± 0.83	688.6 ± 3.74
	-	9.4	71 1 ± 2.76	58.9 ± 3.12	2.91 ± 0.51	536.2 ± 5.55
	-	4.7	60.4 ± 5.12	72. 8 ± 1.93	3.38 ± 0.47	620.5 ± 2.44
LSD ($P \leq 0.05$)			13.1*	3.41*	0.87*	86.4*

Mean values ± standard error (n = 3).
*Significant at $P \leq 0.05$

Alkaloids are constitutively synthesised by members of the Amaryllidaceae family, to which *C. guthrieae* belongs. What role could they possibly be contributing to the overall plant fitness in the absence of a defensive role? From the accumulation patterns observation in this study, it is tempting to speculate that part of their role in this context could be to accumulate and store N for future remobilisation when resources become limiting and likewise for C with phenolic compounds. Alkaloids were generally significantly higher at all C concentrations when N levels were saturating for plant growth. In a related study, **SIMON et al.** (**2010**) investigated N and the growth cost of deploying cyanogenic glycosides in seedlings of *Eucalyptus cladocalyx* by varying N conditions, from levels that were limiting to those that were saturating for growth. The authors found that, for every N invested in cyanogenic glycosides, additional N is added to the leaf and concluded that the growth cost of cyanogenic glycosides was likely too low to detect and that it was offset to some degree by additional N that was allocated alongside the cyanogenic glycosides. For phenolic compounds, in addition to their increased accumulation alongside an increase in C availability, the significantly high levels of total polyphenols, flavonoids and proanthocyanidins per gram of DW accumulated at 175 mM sucrose could be partly attributed to an active metabolic response to osmotic adjustment as opposed to passive correlative accumulation. The previous experiments, demonstrated an increased biosynthesis of this group of compounds in plantlets of the same species subjected to moisture stress (**Section 3.1.3.3**), results of which were consistent with previous findings on other plant species (**ALONSO-AMELOT et al., 2007**). The defence role of polyphenols is thought to work through antioxidant response pathways leading to the protective lignification of smaller polymers that act as antioxidants. Carbon partitioning to constitutive secondary metabolism reportedly increases, and consequently contribute to plant fitness in resource-limited environments (**NCUBE et al., 2012**). The likelihood of interactions between defence mechanisms and growth with the resulting trade-offs between growth and synthesis of defence compounds increases as the two processes compete for limited precursor molecules in resource-constraint environments. Closer comparisons of the two classes of metabolites in this study reveal characteristics in *C. guthrieae* contrary with the proposition of a protein competition model (PCM) (**JONES and HARTLEY, 1999**), with regard to resource partitioning patterns (**Tables 3.3 & 3.4**) as protein

content did not seem to vary markedly across the different C and N concentration ranges compared to alkaloids and polyphenols.

From a medicinal plant perspective, as is the case with *C. guthrieae*, the focus is essentially on how much of the medicinal compounds can be accumulated under a given set of environmental conditions. Polyphenols and alkaloids have diverse medicinal properties. The high levels of the two groups of compounds, particularly at high and low media sucrose concentrations respectively, is of importance for pharmaceutical purposes and offers promising prospects for their enhanced production through nutrient manipulations in culture. *Cyrtanthus guthrieae* could serve as a highly valuable source of alkaloids for pharmaceutical purposes through manipulation of the culture conditions in mass culture cell fermenters and/or bioreactors.

3.3. Effect of Precursor feeding on *in vitro* secondary metabolites

3.3.1. Introduction

The closely co-ordinated series of enzyme-mediated chemical reactions between primary and secondary metabolism in plants are precisely organised to form a network of metabolic pathways through which the synthesis and utilisation of a range of molecular species including sugars and amino acids is achieved. The dividing line between primary and secondary metabolism is indistinct because many of the intermediates in primary metabolism are also intermediates in secondary metabolism. As postulated by **JONES and HARTLEY** (**1999**), the protein competition model (PCM) of phenolic allocation in plants attempts a holistic approach in explaining phenolic biosynthesis on the basis of metabolic origins of pathway constituents, alternative fates of pathway precursors, and the regulatory mechanisms. Protein, phenolic and alkaloid biosynthesis all utilise the phenylalanine ammonia-lyase (PAL)-catalysed committed step with the amino acid phenylalanine (Phe) as a precursor (**DIALLINAS and KANELLIS, 1994**). Following this opinion, protein synthesis competes with secondary metabolites for phenylalanine, leading to a process-level trade-off between rates of synthesis of the two groups of metabolites. Given the assumption that growth takes priority, allocation of resources to secondary

metabolism (phenolics and alkaloids), would therefore, be determined by the competitive dynamics of protein as controlled by the inherent growth demands and environmental cues (**NCUBE et al., 2012**).

In an attempt to exploit the ability of cultured plant cells/tissues to produce useful compounds and to further progress in extending the spectrum of these compounds, this study examines the accumulation patterns of alkaloids and polyphenols through precursor feeding.

3.3.2. Materials and methods

3.3.2.1. Culture treatments and conditions

Cyrtanthus guthrieae plantlets from the optimised micropropagation protocol (**Section 2.2.2**) were cultured in the same MS medium supplemented with 10, 50, 100 and 1000 µM L-phenylalanine and L-tyrosine (Sigma-Aldrich) in a complete randomised design. The cultures were maintained in similar culture conditions as described in **Section 2.2.2** for 6 weeks before samples from each treatment were assayed for alkaloids and polyphenols.

3.3.3. Results and discussion

3.3.3.1. Effect of precursor molecules on secondary metabolism

Tyrosine and Phe serve as precursor molecules in some of the diverse biosynthetic pathways involved in the synthesis of Amaryllidaceae alkaloids as well as polyphenolic compounds (**BASSMAN, 2004**). The treatment with 100 µM Phe induced substantial amounts of callus (**Figure 2.4**, Chapter 2). Compared to whole plantlets, the derived callus recorded lower phytochemical content. This implies that the callus may have a limited capacity to synthesise secondary metabolites due to inadequate cellular development and differentiation. Tissue differentiation plays a significant role in the types of alkaloids produced. For example, the synthesis of vindoline is restricted to the leaves of the plant (**DE LUCA et al., 1988**), while ajmalicine and serpentine are the major alkaloids found in roots of the plant as well

as cell suspension cultures (**VAN DER HEIJDEN et al., 1989**). It has been proposed that secondary metabolite synthesis is associated with young meristematic shoot and root tissues (**PIÑOL et al., 1985; HOBBS and YEOMAN, 1991**), thus lack of or limited cellular differentiation in callus may have reduced the amount of competent tissue for metabolite synthesis. However, it could also be that precursor molecules did not reach the correct cellular or subcellular location necessary for transformation in the multiplying callus.

Table 3.6. Effect of different concentrations of phenylalanine and tyrosine on *in vitro* accumulation patterns of alkaloids and polyphenols in *C. guthrieae*

Treatment (mM)		Phytochemical composition			
		Total alkaloids	Total phenolic content	Flavonoid content	Proanthocyanidin
Precursor	µM	(µg AE g^{-1} DW)	(mg GAE g^{-1} DW)	(µg CTE g^{-1} DW)	(µg LCE g^{-1} DW)
Plantlets					
Phe	10	175.4 ± 7.40^{b}	53.0 ± 6.0^{abc}	2.34 ± 0.10^{d}	358.5 ± 8.0^{c}
	50	243.8 ± 3.91^{d}	69.2 ± 6.7^{cd}	2.87 ± 0.03^{e}	356.0 ± 4.3^{c}
	100	396.8 ± 8.55^{g}	62.9 ± 8.4^{bc}	4.15 ± 0.05^{f}	413.1 ± 4.3^{d}
	1000	352.7 ± 7.56^{e}	107.5 ± 4.5^{e}	4.89 ± 0.11^{g}	430.4 ± 9.4^{d}
Tyrosine	10	133.7 ± 4.15^{a}	43.7 ± 4.0^{a}	1.94 ± 0.10^{c}	324.1 ± 8.7^{b}
	50	488.8 ± 10.95^{h}	55.8 ± 6.1^{abc}	2.17 ± 0.03^{d}	350.9 ± 13.8^{c}
	100	312.0 ± 7.17^{f}	84.0 ± 7.8^{d}	1.65 ± 0.05^{a}	288.8 ± 6.6^{a}
	1000	226.5 ± 8.06^{cd}	52.5 ± 4.9^{abc}	1.68 ± 0.07^{ab}	289.6 ± 5.3^{a}
Callus					
Phe	100	206.1 ± 11.81^{c}	42.6 ± 2.7^{a}	2.16 ± 0.09^{d}	280.2 ± 5.9^{a}
Control	0	118.6 ± 7.60^{a}	45.0 ± 1.9^{ab}	1.89 ± 0.05^{bc}	348.6 ± 1.6^{c}

Mean values ± standard error (n = 3) with different letter(s) in each column indicate significant difference ($P \leq 0.05$) based on DMRT

With regards to alkaloid synthesis in this study, the optimum accumulation (488.8 µg AE g^{-1} DW) was recorded at 50 µM tyrosine (**Table 3.6**). At equimolar concentrations, tyrosine induced significantly higher alkaloid levels at 50 µM than Phe. However, with the exception of 10 µM tyrosine, all treatments had significantly higher alkaloid content than the control. In Phe supplemented media, alkaloid levels increased with the increase in the precursor concentration in the medium up to 100

μM after which it dropped significantly at 1000 μM Phe treatment. A similar characteristic trend of alkaloid accumulation was observed with tyrosine treatments, but with the peak accumulation amount being recorded at 50 μM tyrosine instead. The results indicate 50 μM tyrosine as the optimum and a preferred precursor molecule for *C. guthrieae* under the culture and medium conditions used in this study. Compared to the C-N treatment experiment (**Table 3.5**), media supplements with precursor molecules (Phe and tyrosine) resulted in significantly higher levels of alkaloids in most treatments, an aspect that points to its lucrative potential in mass phytopharmaceutical production in culture.

Polyphenolic compounds, on the other hand, particularly total phenolic content and proanthocyanidins, although significant differences were recorded between most treatments and that of the control, the magnitude of increase was lower compared to those recorded in the C-N treatment experiment (**Table 3.5**). Flavonoids were higher (more than 2-fold) in treatments with 100 and 1000 μM Phe respectively. In light of these results, the question on whether the plant produces these compounds on the basis of relative abundance of precursor molecules or not is raised. The dose-dependent increase characteristic, particularly in alkaloid levels, with the increase in the concentration of precursor molecules in the medium up a certain level, suggest that precursors are expended to synthesise optimum constitutive levels of these metabolites. A sudden decline in alkaloid production when precursor concentrations were increased beyond these optimum levels, points to the existence of metabolic points of regulation and that they probably involve negative feedback inhibition mechanisms. Consistent with these findings, was the increase in podophyllotoxin production following the addition of 3 mM coniferyl alcohol complex as a precursor in *Podophyllum hexandrum* cell cultures, with a 3.7-fold increase in yield on a dry weight basis compared to the control (**WOERDENBERG et al., 1990**). However, the effect of coniferin (another precursor) on podophyllotoxin accumulation was found to be stronger than that of coniferyl alcohol complex (**WOERDENBERG et al., 1990**) at equimolar concentrations. These findings are mirrored with the results of the present study in the case Phe and tyrosine precursors. In addition, metabolic rate-limitations through precursor feeding (**MORGAN and SHANKS, 2000**) and the effect of elicitor dosage (**RIJHWANI and SHANKS, 1998**) on the biosynthesis of indole alkaloids in *Catharanthus roseus* hairy root cultures have been reported. When precursors from

the terpenoid and tryptophan branches were fed to *C. roseu*s root cultures, production of indole-alkaloid increased in a dose-dependent pattern until a dramatic reduction was reached at higher precursor concentration (**MORGAN and SHANKS, 2000**).

Considering that secondary metabolite biosynthesis are elicited in response to plant stress perceptions or developmental cues, an integrated approach that incorporate precursor feeding simultaneously with stress factors can potential increase metabolite yields.

3.4. Conclusions

The increase in the biosynthesis of phenolic compounds with an increase in salt and osmotic stress severity in this study suggests an altered plant metabolism towards accumulation of defence metabolites. Although such metabolic alterations, the magnitude of the response and the type and quantity of metabolites produced under similar stress factor regimes, may vary from one plant species to the next, it suffice to assume, at least in part, to have an altered pool of metabolic compounds. This plasticity in patterns of resource allocation and partitioning also translate to variations in secondary metabolism. This trade-off in resource allocation between primary and secondary metabolism has important implications on the phytomedicinal quality of medicinal plants. The empirical evidences for the significant accumulation of secondary metabolites (phenolic compounds in this study) following plant stress treatments present a potentially promising strategy for improved medicinal properties and value for plants exploited for medicinal purposes like the two *Cyrtanthus* species evaluated in this study. Understanding the defence signalling regulatory pathways and uncovering key mechanisms by which plants tailor their responses to a multitude of stress factors are fundamental in their manipulation towards the desired human benefits. Regulation of these specific factors to levels and duration that favour optimum accumulation of a desired metabolite of interest provides an opportunity for people to utilise plants as chemical factories for producing pharmaceuticals.

The acquisition of NH_4^+ and NO_3^- ions have highly distinct genetic and metabolic consequences in plants, as evidenced by the varied pool of metabolites produced

and differential growth patterns in *C. guthrieae* from this study. The results from *in vitro culture* of *C. guthrieae* plantlets demonstrate that C and inorganic N concentrations in the culture medium determines growth direction and resource partitioning to various metabolites. The plasticity with which metabolism shifted from high/low levels of accumulation of one group of metabolites to another in different media conditions brought about interesting variations in the secondary metabolite pool, an interesting attribute for a medicinal plant species like *C. guthrieae*. While the results offer promising prospects for possible manipulation of the nutrient composition of the growing environments of medicinal plants for a sustained balance between growth and optimal medicinal compound biosynthesis, an integration of such studies with stress factors (abiotic and/or biotic) would likely be a more promising prospect. An integrated approach combining molecular biology, biochemistry and genetic analysis of the gene regulatory networks and mechanisms by which plants respond to C and N ratios would lead to a better understanding of the metabolic interaction between the two macronutrients and thus improve plant productivity.

CHAPTER 4

Pharmacological properties

4.1. Seasonal pharmacological properties and alkaloid content in *Cyrtanthus contractus*

4.1.1. Introduction

Plants synthesise an inconceivable array of diverse chemical structures which, although perceived as waste and detoxification products or the expression of shunt and overflow metabolism, continue to benefit humankind. Alkaloid-containing plants constitute an extremely varied group both taxonomically and chemically, with basic nitrogen being the only unifying factor for the various classes. Throughout history, alkaloids from plant extracts have been used as ingredients in liquid herbal remedies and poisons (**SCINTO et al., 1994; TYLER, 1994**). Prominent examples include Socrates' death in 399 B.C. by the consumption of coniine-containing hemlock (*Conium maculatum*) and Cleopatra's use of atropine-containing extracts of Egyptian henbane (*Hyoscyamus muficus*) during the last century B.C. to dilate her pupils in order to appear more alluring. Medieval European women also utilised extracts of deadly nightshade, *Atropa belladonna*, for the same purpose (**TYLER, 1994; KUTCHAN, 1995**). For this and other reasons, most alkaloids have been identified as extremely toxic and alkaloid containing plants do not feature prominently in herbal medicine but have always been important in the allopathic system where dosage is strictly controlled, and in homoeopathy where dose-rates are so low as to be harmless. However, despite the extreme toxic properties of some alkaloids to find therapeutic uses outside that of homeopathy, tropicamide, an anticholinergic synthetic derivative of atropine, is routinely used in eye examinations to dilate the pupil and has also shown potential as an early diagnostic tool in the detection of Alzheimer's disease (**SCINTO et al., 1994**).

The physiological role of alkaloids in plants, their importance in taxonomy, and biogenesis are often discussed at the level of a particular class of alkaloid. Being products of secondary metabolism with ecological adaptive roles (**HARTMANN,**

1991), their synthesis and accumulation in plants is influenced by seasonal changes in environmental conditions within the growing environments (**NCUBE et al., 2012**). The inherently unique capability of plants to alter the pattern of alkaloid accumulation through gene activation and expression patterns can be exploited through manipulation of environmental factors. The expression patterns of often complex and long biosynthetic pathways of pharmaceutically important plant alkaloids, can only be effectively exploited under natural environmental fluctuations. A number of complex plant alkaloid structures of pharmaceutical importance such as codeine, vinblastine, taxol, and camptothecin remain well beyond the reach of commercially feasible total chemical syntheses (**KUTCHAN, 1995**), an attribute that positions natural plant populations as valuable sources of these compounds. Even with the development of cell culture technology, is has been recognised that some compounds are not synthesised to any appreciable extent in culture (**RAMACHANDRA RAO and RAVISHANKAR, 2002; TIKHOMIROFF and JOLICOEUR, 2002; VANISREE et al., 2004**). The reason is thought to lie in the tissue-specific expression of their biosynthetic genes.

Among the various natural sources of alkaloids that have been investigated for potential use in cancer treatments, members of the Amaryllidaceae family have been particularly fruitful (**HARTWELL, 1969**). The ethnobotanical aspects and medicinal usage of the Amaryllidaceae plants are rooted in their unique alkaloid constituents (**BASTIDA et al., 2006; JIN, 2009**). To date, more than 100 structurally diverse alkaloids, possessing a wide spectrum of biological activities have been isolated from various Amaryllidaceae species (**HOSHINO, 1998**). The genus *Cyrtanthus* Aiton is endemic to sub-Saharan Africa, with well over 90% of the species concentrated in South Africa (**REID and DYER, 1984**). As a consequence of the impressive distribution of compounds within *Cyrtanthus* species which interact effectively with enzymes and pathogenic microbes (**ELGORASHI and VAN STADEN, 2004; EVIDENTE et al., 2009; NAIR et al., 2011**), evaluating their pharmacological properties with changing seasons has potential for identification of new pharmaceutical leads.

4.1.1.1. Bacterial and fungal infections and treatments

As infectious diseases evolve and pathogens develop resistance to existing pharmaceuticals, the search for new novel leads, possibly with different modes of action against fungal, bacterial, and viral diseases has intensified over recent years (**GIBBONS, 2004; AVILA et al., 2008**). Antibiotics are used as antibacterial agents for the treatment of infectious diseases. However, this important resource is often scarce in most developing countries and for this reason infectious diseases are still a significant cause of mortality and morbidity for these populations (**HART and KARIUKI, 1998**). Plants offer a unique resource that provides a diverse array of natural products, which people exploit in the treatment of a range of ailments in most developing communities.

Bacteria forms one of the most abundant groups of unicellular organisms capable of adapting to a diverse range of environments (**SLEIGH and TIMBURY, 1998**). The bacterial group consists of microorganisms that are both useful and harmful to humans. Although not all bacterial species are harmful, bacterial infections are widespread and ranked as one of the major causes of death worldwide (**SLEIGH and TIMBURY, 1998; ROTUN et al., 1999; AVILA et al., 2008**).

Bacillus subtilis, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* are among the numerous medically important bacteria responsible for a number of infectious diseases. *Staphylococcus aureus* is a Gram-positive commensal bacterium that is commonly cited as a major hospital-acquired pathogen (**ROTUN et al., 1999; RUBIN et al., 1999**). The bacterium is a causative agent for such infections as, septic arthritis, boils, conjunctivitis, endocarditis, pneumonia, skin infections, meningitis, wound infections and food poisoning (**SLEIGH and TIMBURY, 1998; RUBIN et al., 1999**). The ability of the strains of *S. aureus* to acquire resistance to practically all useful antibiotics (**GIBBONS, 2004**) is a cause for considerable concern in medical circles. *Bacillus subtilis* is a soil saprophyte, endospore-forming Gram-positive bacterium. It causes food poisoning and occasionally, some opportunistic infections such as conjunctivitis (**MURRAY et al., 1998; RYAN and RAY, 2004**). *Escherichia coli*, a Gram-negative bacterium is present in mammals as part of the normal flora of the gastrointestinal tract. The

bacterium is also a causative agent of infections such as, urinary tract and wound infections, bacteriaemia and pneumonia (**SLEIGH and TIMBURY, 1998**). The non-motile Gram-negative *K. pneumoniae* is responsible for urinary tract infections, septicaemia, meningitis and pneumonia in humans (**EINSTEIN, 2000**).

Besides bacterial infections, humans are also battling with the challenges of fungal infections. The number of deaths due to fungi largely surpasses that by parasites in developed countries (**LATGÉ and CALDERONE, 2002**). As opposed to bacteria, very few fungal pathogens are causative agents of infectious diseases. *Candida albicans*, forms part of the normal flora of the respiratory, gastrointestinal and female genital tracts in humans and is responsible for up to 90% of fungal infections (**CHAITOW, 1996**). Immunosuppression is the key factor that triggers the establishment and dissemination of fungal infections. The establishment of an infection by a fungal pathogen and its invasion and growth in host tissues requires that the fungus is aggressive at a time when the host's immune response is debilitated (**LATGÉ and CALDERONE, 2002**). The opportunistic pathogens are normally eradicated by the innate immunity of the immunocompetent host. Up to 90% of all HIV/AIDS patients suffer fungal infections at some point during the course of the disease, with up to 20% of them dying as a direct consequence of such infections (**DIAMOND, 1991; CHAITOW, 1996**). In HIV/AIDS patients, *C. albicans* leads to the development of oral candidiasis, which progresses to the oesophagus to prevent intake of adequate oral nutrition, resulting in increased morbidity and mortality (**REICHART, 2003**).

Pathogenic bacterial strains vary in their susceptibility to antibiotic treatments. For example, most strains of *Streptococcus pneumoniae* are inhibited by 0.01 μg^{-1} ml of benzylpenicillin whereas with *E. coli*, a concentration of 32-64 μg^{-1} ml is required to inhibit growth, a level which cannot be achieved within the body (**HAWKEY, 1998**). Broad spectrum antibiotic agents such as cephalosporins and fluoroquinolones have been developed for the blanket treatment of a number of bacterial infections. The widespread use of broad spectrum agents coupled with misuse and various other factors have tilted the delicate balance between humans and bacteria. As a result, pathogens have developed resistance to antimicrobials. The limited number of efficacious drugs available clinically to treat fungal infections (**RUHNKE et al., 1994**)

presents a major challenge. Novel alternatives to treating fungal infections are required to circumvent the often poorly effective but expensive fungal treatments. Resistance to antibiotics constitutes a major threat to public health and has become a global concern. The search for alternative agents is therefore inevitable.

4.1.1.2. Enzyme inhibition by natural products

The immune system of the body is composed of many interactive, specialised cell types and enzymes that collectively protect the body from pathogenic infections and other sources of stimuli. Enzymes are involved in virtually every catalytic process in the body, and as such are the most attractive targets for drug intervention in the treatment of a number of human conditions. Enzyme inhibitors represent almost half the drugs in modern clinical use (**COPELAND, 2005**).

Inflammation is one of the first responses of the immune system to infection involving the recruitment of immune cells to the site of injury. An inflammatory reaction serves to establish a physiological barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of the stimuli (**COHN and LANGMAN, 1996**). According to **FERRERO-MILIANI et al.** (**2006**), inflammation is thus defined as a localised protective, non-specific immune response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. The complex process of inflammation begins with the mobilisation of arachidonic acid (AA) from the membrane phospholipids through the action of the enzyme phospholipase A_2 (PLA_2) following stimulation (**PATRIGNANI et al., 2005**) (**Figure 4.1**). The liberated AA serves as the substrate to the rate limiting step in prostanoid biosynthesis, catalysed by cyclooxygenase (COX) enzymes. Prostanoids are lipid mediators that coordinate a wide range of physiologic and pathologic processes via membrane receptors on the surface of target cells (**FITZPATRICK and SOBERMAN, 2001**). Their broad spectrum of effects embraces practically every biological function as well as mediation of pain, fever and swelling during inflammation. Under physiologic conditions, prostanoids play an important role in the cytoprotection of the gastric mucosa, haemostasis, and renal haemodynamics (**PATRIGNANI et al., 2005**). COX enzymes catalyse the transformation of AA into an unstable prostaglandin H_2 (PGH_2) from which all other prostanoids are metabolised

through isomerases with different structures and exhibiting cell- and tissue-specific distribution (**PATRIGNANI et al., 2005**).

The expression of the two COX isozymes (COX-1 and COX-2) is differently regulated but the enzymes are moderately similar in their amino acid sequences. The constitutive COX-1 enzyme is expressed in a wide variety of tissues while COX-2 is induced almost exclusively in inflamed tissues responding to inflammatory stimuli such as proinflammatory cytokines, growth factors, mitogens, bacterial lipopolysaccharide, and tumour promoters (**FLETCHER et al., 1992**). COX-2 is thought to contribute to the generation of prostanoids at sites of inflammation (**CROFFORD et al., 2000**). Prostanoid biosynthesis is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) widely prescribed as analgesics and anti-inflammatory agents. Their mechanism of action, particularly aspirin and ibuprofen involves the inhibition of both COX-1 and COX-2 isozymes thereby preventing the pathological synthesis of prostaglandins (**MORITA, 2002**). However, inhibition of COX-1 result in the removal of the cytoprotection function, leading to damaging side effects in the gastrointestinal tract (**GILROY et al., 1998**). The undesirable side effects associated with the use of NSAIDs through inhibition of COX-1 has stimulated research interest in alternative forms of anti-inflammatory agents. Selective inhibition of COX-2 enzymes has been shown to significantly lower the development of gastro-toxicity and related side effects (**FERRERO-MILIANI et al., 2006**). Plants, therefore, offer a vast biogenic resource base for exploitation in the discovery of innovative anti-inflammatory agents.

Another pharmacologically important enzyme in drug development is acetylcholinesterase (AChE), which catalyses the hydrolytic degradation of the neurotransmitter acetylcholine, with choline and an acetate group as end products. AChE is found at neuromuscular junctions and cholinergic synapses in the central nervous system (CNS), where its catalytic activity serves to halt synaptic transmission (**VOET and VOET, 1995**). The malfunctioning of biochemical pathways involved in the production of the neurotransmitter acetylcholine leads to progressive memory loss and cognitive impairment (**PANG et al., 1996**), a condition known as Alzheimer's disease (AD). Treatment of AD takes advantage of AChE inhibition. Galanthamine, an alkaloid isolated from snowdrop (*Galanthus nivalis*, in the family

Amaryllidaceae), is a potent inhibitor of AChE widely used for AD treatments (**HOUGHTON et al., 2006**). Galanthamine is a selective, reversible and competitive inhibitor of AChE and an ideal chemotherapeutic agent for the treatment of the neurodegenerative disease (**HOUGHTON et al., 2006**). Inhibitors of AChE are also employed in the treatment of Myasthenia gravis, an autoimmune disorder, characterised by severe muscle weakness caused by circulating antibodies that block acetylcholine receptors at the post-synaptic neuromuscular junction, inhibiting the stimulative effect of acetylcholine (**ROSENBERRY, 2006**).

The use of natural plant-derived products in the treatment of pain, cancer, infectious diseases and neurodegenerative disorders holds tremendous potential owing to the unique ability of plants to carry out *in vivo* combinatorial chemistry by shifting and matching gene products in response to environmental stimuli. Due to its widespread use in traditional medicine and alkaloid content, outdoor growing populations of *Cyrtanthus contractus* were evaluated for their seasonal response in alkaloid content, antimicrobial activity, cytotoxic properties as well as inhibitory effects against COX-1 and COX-2 and AChE enzymes.

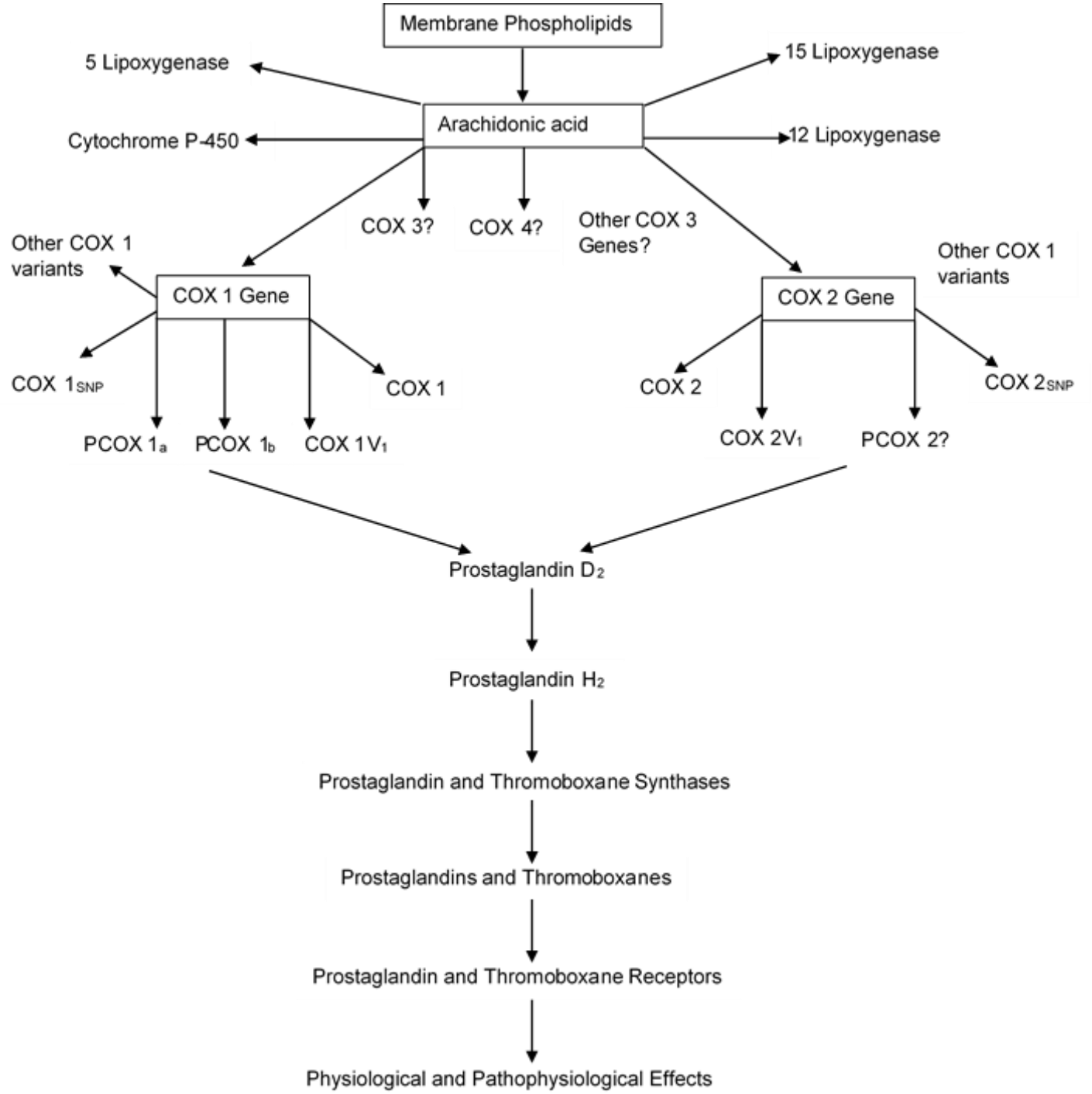


Figure 4.1. Schematic pathways of prostaglandin and thromboxane formation in the inflammation process (**DAVIES et al., 2004**)

4.1.2. Materials and methods

4.1.2.1. Plant material and extract preparation

Bulbs from a naturally growing population of *Cyrtanthus contractus* in a grassland area adjacent the School of Life Sciences at the University of KwaZulu-Natal (UKZN), Pietermaritzburg were randomly collected on the 15th day of each month beginning from October 2011 to September 2012. A voucher specimen (**NCUBE 05 NU**) identified by Dr. Christina Potgieter was deposited at the Bews Herbarium (NU) at UKZN, Pietermaritzburg. The bulbs from each monthly collection sample were dried in an oven at a constant temperature of 50 °C, ground into a fine powder using an Ultra Centrifugal Mill (ZM 200, Retsch®, Germany) and stored in airtight containers in the dark at room temperature. The ground samples were extracted by maceration with 20 ml^{-1} g of ethanol in a sonication bath (Julabo GMBH, Germany) containing ice for 2 h. The crude extracts were then filtered under vacuum through Whatman's No. 1 filter paper. The extracts were then concentrated *in vacuo* at 35 °C using a rotary evaporator (Rotavapor-R, Büchi, Switzerland) and the dry extracts kept in airtight glass sample vials at 10 °C in the dark until they were used for the different assays

4.1.2.2. Alkaloid quantification

Samples of extracts obtained from **Section 4.1.2.1** were used for the estimation of total alkaloid content of each sample following the procedure described in **Section 3.2.2.7.**

4.1.2.3. Cytotoxicity test

4.1.2.3.1. Cell culture

Stock solutions (15 mg^{-1} ml) of sample extracts (**Section 4.1.2.1**) and controls, Galanthamine and Staurosporine (10 mmol^{-1} l) were prepared in dimethylsulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, foetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (MO, USA). Calcein AM was obtained from Molecular Probes

(Invitrogen Corporation, CA, USA). Test cell lines (T-lymphoblastic leukemia CEM, breast carcinoma MCF7, cervical carcinoma HeLa and human fibroblasts BJ) were obtained from the American Type Culture Collection (ATCC) (Manassas, USA). All cell lines were cultured in DMEM medium (Sigma-Adrich, USA), supplemented with 10% heat-inactivated foetal bovine serum, 2 $mmol^{-1}$ l L-glutamine, 10 000 U penicillin and 10 mg^{-1} ml streptomycin. Cell lines were maintained under standard cell culture conditions (37 ºC and 5% CO_2 in a humid environment), and were sub-cultured twice or thrice weekly as required according to standard trypsinisation procedures (**MAJ et al., 2011**).

4.1.2.3.2. Calcein AM assay

Cell line suspensions (ca. 1.0×10^5 $cells^{-1}$ ml) were placed in 96-well microtitre plates and after 24 h stabilisation (time zero), sample extracts which were serially diluted in DMSO were added (20 µl aliquots) in four replicates. Control cultures were treated with DMSO alone, such that the final DMSO concentration in the incubation mixtures never exceeded 0.6%. Extracts were evaluated at six 3-fold dilutions with the highest final concentration of 100 μg^{-1} ml. After 72 h incubation under standard cell culture conditions (37 ºC and 5% CO_2 in a humid environment), 100 µl Calcein AM solution (Molecular Probes, Invitrogen, CA, USA) was added, and incubation continued for a further 1 h. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values were calculated (μg^{-1} ml).

4.1.2.4. Acetylcholinesterase (AChE) enzyme inhibitory bioassay

Inhibition of AChE by the plant extracts was done as described by **ELLMAN et al.** (**1961**) with slight modifications. The acetylcholinesterase enzyme activity was measured by spectrophotometric observation of the increase in a yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. The following buffers were used; Buffer A: 50 mM Tris-HCl, pH 8; Buffer B: 50 mM Tris-

HCl, pH 8, containing 0.1% bovine serum albumin (BSA); Buffer C: 50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$. Acetylthiocholine iodide (ATCI), galanthamine, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and AChE (isolated from electric eels) (type VI-S lyophilized powder) were obtained from Sigma-Aldrich.

In a 96-well plate, 25 µl of each sample extract at an initial concentration of 10 mg^{-1} ml were added to the first well and serially diluted two-fold down the plate. To the diluted sample, 25 µl of 15 mM ATCI in water, and 125 µl of 3 mM DTNB in buffer C were added, followed by 50 µl of buffer B. The absorbance of the reaction mixture was then measured three times at 405 nm every 45 s using a microplate reader (ChroMate 4300, Awareness Technology Inc.). After the third reading, 25 µl of 0.2 U^{-1} ml AChE in buffer A was added. The absorbance was measured again every 45 s for a further five times. The final concentration of the plant extracts in the first well containing the highest concentration was 1.0 mg^{-1} ml. Galanthamine at 0.12, 0.23, 0.46, 0.92, 1.84, 3.68 and 7.37 µg ml^{-1} concentrations and water were used as positive and negative controls respectively. The increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the ratio of reaction before adding the enzyme from the rate after adding the enzyme. Percentage of inhibition was calculated by comparing the reaction rates for the sample to the negative control. Results were presented as means ± standard errors of the experiment in duplicate. The IC_{50} values of herbal preparations were calculated using Graph Pad Prism (version 5.0) statistical software programme for Windows (GraphPad Software Inc.).

4.1.2.5. Cyclooxygenase-1 (COX-1) inhibitory bioassay

The COX-1 inhibitory activity of plant extracts were evaluated as described by **WHITE and GLASSMAN** (**1974**) and modified by **JÄGER et al.,** (**1996**). Plant extracts, 2.5 µl aliquots (10 mg^{-1} ml) were diluted with 17.5 µl distilled water in 1.5 ml Eppendorf tubes. Stock solution of COX-1 enzyme stored at -70 °C was activated with 1450 µl of co-factor solution (0.3 mg^{-1} ml L-epinephrine, 0.3 mg^{-1} ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, at pH 8.0) and pre-incubated on ice for 5 min. The enzyme/co-factor solution (60 µl) was then added to each Eppendorf tube with sample extracts in duplicate and the mixture incubated at room

temperature for 5 min. The substrate, (20 µl) [^{14}C]-arachidonic acid (17 Ci mol^{-1}, 30 µM), was added to each tube to initiate the enzymatic reaction and the preparations were incubated in a water bath at 37 ºC for 10 min. The final concentration of extract samples in the bioassay was 250 µg ml^{-1}. After incubation, the reaction mixtures were placed on ice and the reaction terminated by adding 2N HCl (10 µl) in each reaction tube. Four microlitres (0.2 mg^{-1} ml) of unlabelled prostaglandins (PGE_2: $PGF_{2\alpha}$; 1:1) (Sigma-Aldrich) were added to each Eppendorf tube as a carrier solution.

Glass columns were packed with silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pasteur pipettes. Prostaglandins were separated from unmetabolised arachidonic acid by applying the test mixtures to the columns with 1 ml of eluent 1 [hexane: 1, 4-dioxane: glacial acetic acid (70:30:0.2 v:v:v)]. Unmetabolised arachidonic acid was eluted first with 4 ml of eluent 1. The prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15; v:v)] and collected into scintillation vials. To each vial, 4 ml of scintillation fluid (Beckman Coulter^{TM}, USA) was added and the disintegration per minute of the radioactive material was counted using a LS 6500 multipurpose scintillation counter (Beckman Coulter^{TM}, USA). Controls for the assay, in duplicate, included a solvent blank, background and an indomethacin standard. The solvent blank and background consisted of 70% ethanol (2.5 µl) instead of sample extracts. The background controls were kept on ice throughout the assay and the enzyme was inactivated using 2N HCl (10 µl) before adding [^{14}C]-labelled arachidonic acid. Indomethacin (2.5 µl) (positive control) was used at a concentration of 5 µM. The assay was repeated twice. The percentage of prostaglandin synthesis inhibition by the plant extracts were calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank and background samples using the equation below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}\right)\right\} \times 100$$

where DPMextract, DPMbackground and DPMsolvent blank represent the disintegrations per minute for sample extract, background and solvent blank respectively.

4.1.2.6. Cyclooxygenase 2 (COX-2) inhibitory bioassay

The COX-2 inhibitory activity was assessed using a method described by **NOREEN et al.,** (**1998**) with slight modifications (**ZSCHOCKE and VAN STADEN, 2000**). The assay followed a similar experimental procedure as described for the COX-1 bioassay except that for the preparation of the co-factor solution, 0.6 mg^{-1} ml L-epinephrine was used in place of 0.3 mg^{-1} ml and 200 µM Indomethacin instead of 5 µM. The rest of the assay was as described for COX-1 (**Section 4.1.2.5**).

4.1.2.7. Antimicrobial bioassays

4.1.2.7.1. Preparation of microbial stock cultures

Bacterial stock strains used for the antibacterial bioassays were cultured in Mueller-Hinton (MH) agar (Merck, Germany). Sterile (25 ml) MH was poured into plastic Petri dishes and allowed to gel. The plates were allowed to cool overnight at 4 °C after which stock strains of the bacteria were streaked and sub-cultured. The bacteria-inoculated plates were incubated at 37 °C for 24 h to allow bacterial colonies to develop. The plates were then stored at 4 °C to prevent further bacterial growth. The stocks were sub-cultured following the same procedure every 30 days in order to maintain viability. A similar procedure was followed for the maintenance of *C. albicans* with Yeast Malt (YM) agar (Becton Dickinson, USA) used in place of MH agar.

4.1.2.7.2. Antibacterial microdilution bioassay

Minimum inhibitory concentration (MIC) values for the antibacterial activity of the plant extracts were determined using the microplate dilution technique (**ELOFF, 1998**). One hundred microlitres of each resuspended extract (25 mg ml^{-1} in 70% ethanol) were two-fold serially diluted with sterile distilled water, in duplicate, down

the 96-well microtitre plate (Greiner Bio-one GmbH, Germany) for each of the four bacteria strains used. A similar two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) (0.1 mg ml^{-1}) was used as a positive control for each bacterium. Distilled sterile water and 70% ethanol were included as negative and solvent controls respectively. Overnight MH (Oxoid, England) broth cultures (incubated at 37 °C in a water bath with an orbital shaker) of four bacterial strains: two Gram-positive (*B. subtilis* American type culture collection (ATCC) 6051 and *S. aureus* ATCC 12600) and two Gram-negative (*E. coli* ATCC 11775 and *K. pneumoniae* ATCC 13883) were diluted with sterile MH broth to give a final inoculum of approximately 10^6 cfu^{-1} ml (colony forming units). One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was indicated by adding 50 µl of 0.2 mg^{-1} ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a further incubation at 37 °C for 1 h. Since the colourless tetrazolium salt is biologically reduced to a red product due to the presence of active organisms, the MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. Bacterial growth in the wells was indicated by a reddish-pink colour. The assay was repeated twice.

4.1.2.7.3. Anticandidal microdilution bioassay

A microdilution method as described by **ELOFF** (**1998**) and modified for fungi (**MASOKO et al., 2007**) was used to determine the anticandidal activity of the extracts against *C. albicans* (ATCC 10231). An overnight fungal culture was prepared in YM (Oxoid, England) broth. Four hundred microlitres of the overnight culture were added to 4 ml of sterile saline (0.85% NaCl) and the absorbance was read at 530 nm using a UV-vis spectrophotometer (Varian Cary 50, Australia). The absorbance was adjusted with sterile saline to match that of a 0.5 M McFarland standard solution of between 0.25-0.28 absorbance units. From this standardised fungal stock, a 1:1000 dilution with sterile YM broth was prepared giving a final inoculum of approximately 10^6 cfu^{-1} ml.

One hundred microlitres of each resuspended extract (50 mg ml^{-1}) were serially diluted two-fold with sterile water in a 96-well microtitre plate. A similar two-fold

dilution of Amphotericin B (Sigma-Aldrich, Germany) (2.5 mg ml^{-1}) was used as the positive control while sterile distilled water, fungal free YM broth and 70% ethanol were used as negative and solvent controls respectively. One hundred microlitres of the dilute fungal culture were added to each well. The plates were covered with parafilm and incubated at 37 °C for 24 h after which 50 µl (0.2 mg ml^{-1}) INT were added and incubated for a further 24 h at 37 °C. The wells remained clear where there was inhibition of fungal growth. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48 h. To determine the fungicidal activity, 50 µl of sterile YM broth was added to all the clear wells, covered with parafilm and further incubated at 37 °C for 24 h after which the minimum fungicidal concentrations (MFC) were recorded as the last clear wells. The assay was repeated twice with two replicates per assay.

4.1.2.8. Statistical analysis

Data on alkaloid content was subjected to one-way analysis of variance (ANOVA) using GenStat 14th edition (VSN International, UK). Significantly different means were separated using Least Significant Difference (LSD) technique ($P \leq 0.05$) and results presented as (mean ± standard error).

4.1.3. Results and discussion

4.1.3.1. Alkaloid variations

Sessile organisms, plants, constantly interact with changing biotic and abiotic conditions such as temperature, light intensity, moisture stress, herbivory and microbial attack. As an adaptive strategy to survive under potentially damaging conditions, plants generate chemical defence mechanisms which spans many complex biochemical processes (**HOLOPAINEN and GERSHENZON, 2010**). In recognition of the fact that environmental factors have an especially profound influence on plant secondary metabolism, weather data (rainfall and temperature) in the area of plant collection were recorded on a daily basis during the collection period (October 2011-September 2012) and presented as an average for each

month (**Table 4.1**). Alkaloids, particularly the Amaryllidaceae group, are of important pharmacological value in modern and parallel medicinal systems, with a range of reported biological activities (**ROBEY et al., 2001; GRIFFIN et al., 2007; VAN GOIETSENOVEN et al., 2010**).

The total alkaloid content in *C. contractus* bulbs collected during different months of the year are presented in **Figure 4.1**. Although no noticeable distinct trend was observed, significantly higher alkaloid levels (1020.6 µg g^{-1} DW) from the bulb extracts were recorded in the month of September followed by those collected in January (955.4 µg g^{-1} DW). The least amount of alkaloid was recorded in the month of November. A closer comparison of weather parameters (**Table 4.1**) with alkaloid content (**Figure 4.1**) during these time periods reveals that in the month of September, temperatures ranged between 7 °C and 33 °C with an average of 16.9 °C and at least 8 days of over 25 °C. An average of 123 mm of rainfall was received for the month, with about 13 days of complete overcast weather conditions. On the other hand, in the month of January, the temperature range was 17-38 °C with an average of 25.5 °C and more than 23 days of over 25 °C and 10 days of over 30 °C. The rainfall amounted to 79 mm for the month occurring in only 4 days with 3 days of complete overcast weather conditions. Although no generalisations on the effect of temperature on the alkaloid levels could be made, however, based on these contrasting sets of data, it is tempting to speculate that high temperatures, particularly those experienced in the month of January could have had an influence on the surge in alkaloid production of *C. contractus* bulbs during this period. Numerous studies report alkaloid levels to be influenced by seasonal variations in temperature in some plant species (**PRESTIDGE et al., 1985; BREEN, 1992; SALMINEN et al., 2005**). In a study on the influence of temperature on alkaloid levels in tall fescue and perennial ryegrass, **SALMINEN et al.** (**2005**) reported the components; ergonovine, chanoclavine, and perloline, to be significantly influenced by temperature, whereas ergovaline was not. Although the authors concluded that temperature had a significant influence on overall alkaloid expression in both grasses, the influence of temperature on individual alkaloids varied over time. The variations in alkaloid content on a month to month basis observed in this study might probably be explained, in part, on a similar basis, in that biosynthesis of different

classes of alkaloids are upregulated and/or downregulated at different times and plant phenological cycles as dictated by environmental cues (**RÍOS et al., 2013**). Apart from the high temperatures and rainfall coinciding with high alkaloid production levels in September, the same period also happens to be the onset of the flowering period for the species. The large investment in nitrogen (N) by alkaloid producing plants has been attributed to the ecological role of these chemical compounds to plant adaptation (**FACCHINI, 2001**) as well as in regulating plant growth (**ANISZEWSKI, 2007**). Following this logic, the surge in alkaloid levels during September and January, in the present study, could be attributed to the specific group of alkaloids involved in such growth regulatory functions as demanded by the plant during these time periods of the species growth cycle. In September, for example, a role in flowering could be assumed. Similarly, the monthly fluctuations in alkaloid levels in *C. contractus* reported in this study could be interpreted as reflections of ecological adaptations to changing environmental regimes, growth and developmental demands.

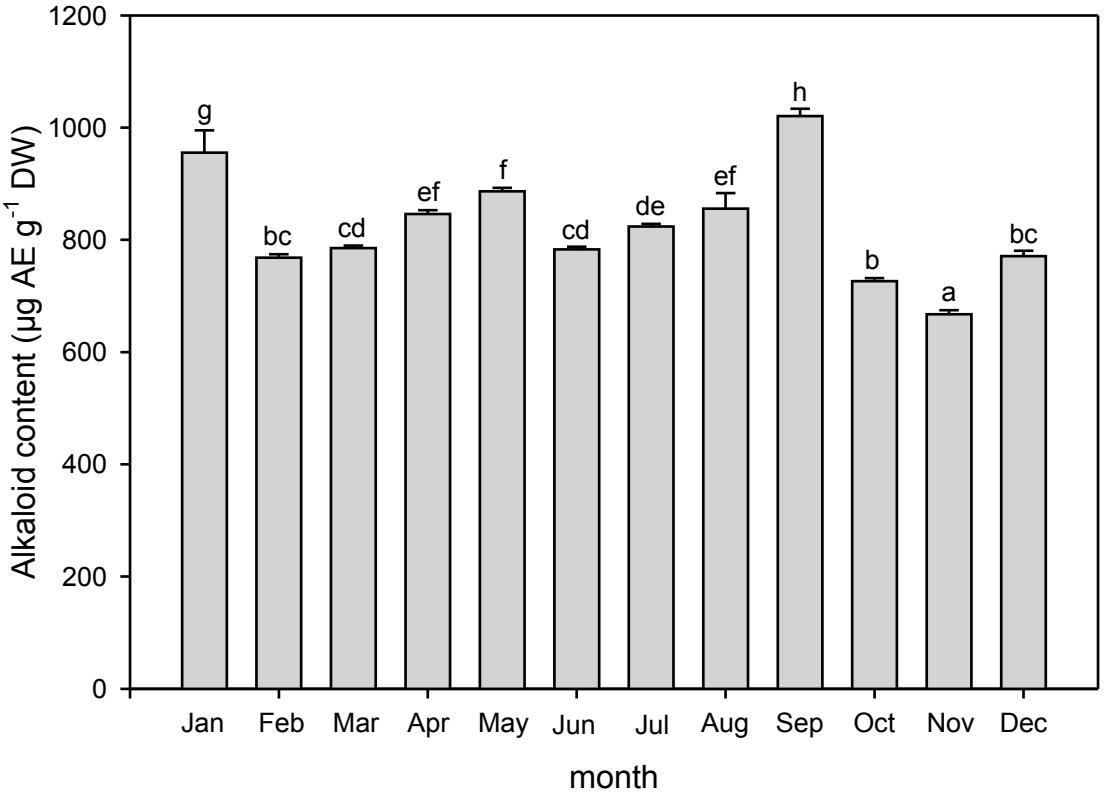


Figure 4.2. Total alkaloid content of *Cyrtanthus contractus* in the different months of the season

A chemical study conducted along with phenological cycles in *Lapiedra martinezii* populations (**RÍOS et al., 2013**), revealed slight changes in the alkaloid patterns in the plants at the flowering stage, when the populations had no leaves, compared to those found during the vegetative growth stage. In the same study, a trend of decreasing lycorine-type and increasing homolycorine-type alkaloids was observed in the bulbs during the flowering stage, with the amount of tazettine-type alkaloids in the bulbs reaching up to 24%. The same type of alkaloid also appeared during vegetative growth (**RÍOS et al., 2013**). This kind of correlative fluctuations in the types of alkaloid relative to the producing plant growth stages further affirms to and reinforces their purported regulatory functions in plant growth. Although inexplicable, as it may largely be interpreted, **SEIGLER and PRICE** (**1976**) emphasise an ecological context of function as opposed to growth regulatory role of these groups of compounds. In explaining the fluctuating levels of secondary metabolite production under different light regimes, **DIALLINAS and KANELLIS** (**1994**) and **LIU et al.** (**2006**) highlight the influence of light on most of the enzymes involved in secondary metabolism. In particular, the activity of phenylalanine ammonia-lyase (PAL), a key enzyme in the biosynthetic pathway of alkaloids, increases under the influence of light (**SEIBERT and KADKADE, 1980**). The increase in alkaloid accumulation with illumination of *Scopolia parviflora* cultures reported by **TABATA et al.** (**1971**) was mirrored in the results obtained latter by **HOBBS and YEOMAN** (**1991**) in tobacco, although **SEIBERT and KADKADE** (**1980**) reported a complex relationship between light intensity and spectral quality on the accumulation of this group of compounds, with defined light regimes causing inhibition in cultured segments of *Helianthus tuberosus* (**GAUTHERET, 1969**). Although the evidence for the adaptation/defensive role of alkaloids is impressive, other selective advantages for these compounds exist (**SEIGLER and PRICE, 1976; KUTCHAN, 1995; RÍOS et al., 2013**) and it is thus logical to assume that these multidimensional aspects could have been involved in shaping the patterns of alkaloids observed in this study.

Constitutive defence mechanisms differentially respond to the various environmental cues but most importantly, upon exposure to a set of different environmental regimes, induced metabolic responses take effect. Induced defence responses are characterised by alterations in a set of traits that lead to a reduction in the negative effect of the stress factor(s) on plant fitness. Throughout the course of growth and

development, plants are exposed to various biotic and abiotic factors to which they respond with an activation of the defence system, hence variations in these chemical constituents to changing environmental conditions. Environmental factors, together with the plant's morphological characteristics at different developmental stages usually play a major role in determining the quantity and quality of the plant's chemical constituents (**RICHARDSON et al., 2004; VILJOEN et al., 2005**). These factors, however, change markedly with changing seasons and could therefore account for the differences in the extract constituents. From a traditional medicine point of view, matching collection of medicinal plant material with peak bioactive compound production and optimum biological activity is an important strategy for improved medical benefits.

Table 4.1. Average monthly data on temperature and rainfall patterns from the collection site for *Cyrtanthus contractus* during the period October 2011 to September 2012. GPS location (29.625° S, 30.403° N)

Year	Month	Temp Min (°C)	Temp Max (°C)	Average Temp (°C)	No of days > 25	No of days >30	No of days <10	No of days < 5	Rainfall (mm)	No of rainy days	No of overcast days
2011	Oct	10	33	19.7	15	5	-	-	49	10	9
	Nov	10	38	21.2	16	6	-	-	92.5	9	10
	Dec	14	38	25.1	27	12	-	-	140.5	15	6
2012	Jan	17	38	25.5	23	15	-	-	79	4	3
	Feb	18	35	25.6	27	19	-	-	24.5	6	6
	Mar	12	35	22.2	21	5	-	-	121	11	11
	Apr	8	32	18.4	9	1	2	-	17	4	7
	May	4	33	17.6	10	1	14	3	4.5	2	2
	Jun	1	26	12.7	1	0	30	9	15	2	6
	Jul	3	26	14.4	2	0	30	6	9	1	7
	Aug	3	35	16.2	11	4	13	5	80	4	9
	Sept	7	33	16.9	8	2	4	0	123	8	13

4.1.3.2. Cytotoxicity effects

Bulb extracts collected in the month of May decreased the survival of all cancer cell lines in a dose-dependent pattern, with IC_{50} values ranging from 11.7 to 37.9 µg ml^{-1} after 72 h of treatment (**Table 4.2**). The extracts from bulbs collected in September also resulted in a survival decrease in all cell lines after 72 h although, on a cell by cell comparison, the IC_{50} values were higher than those obtained from the extracts collected in May. This study considered extracts with IC_{50} values <100 µg ml^{-1} to have effective cytotoxic effects. Among the four cell lines that showed susceptibility to the potent plant extracts, T-lymphoblastic leukaemia cells (CEM) were found to be the most sensitive with IC_{50} ranges of between 11.7 to 72.3 µg ml^{-1}. On a month to month comparison, the extracts obtained in June and November demonstrated the least cytotoxic effects against all cancer lines compared to those from other months. A closer comparison between the extracts obtained in June with those from the preceding month (May) reveals marked differences in IC_{50} cytotoxicity values. Similarly, the extracts from November samples with those of the preceding month (October) or proceeding one (December), showed significant difference in their cytotoxic effects against most cancer cell lines. This sharp contrast in extract activity despite the temporal proximity between these months, points to an abrupt shift in the concentration and/or balance thereof of the active constituents within the extracts. Regarding AChE inhibitory activity, a similar trend to that of cytotoxicity was observed between the extracts from the two months (May and June). A snap look at the alkaloid content between the two months in question, reveals that alkaloid levels were significantly higher in May than in June. However, compared to the other two months with higher alkaloid levels than in May (January and September), the extracts obtained in the month of May maintained the best cytotoxic effect against all tested cell lines than these other two months. The results suggest extract quality rather than mere quantity to be a major contributing factor to this observed trend in activity between the different months.

Table 4.2. Acetylcholinesterase inhibitory activity and cytotoxic effects of seasonally collected *Cyrtanthus contractus* bulb extracts

Plant extract	AChE inhibitory activity		Cytotoxicity activity IC_{50} (µg ml^{-1})			
	(%) Inhibition	IC_{50} (µg ml^{-1})	CEM	MCF7	HeLa	BJ
January	68.5 ± 2.7	98.4 ± 1.4	16.0 ± 0.1	>100	83.3 ± 8.2	35.6 ± 7.0
February	55.3 ± 3.4	633.4 ± 4.1	62.4 ± 10.8	>100	>100	>100
March	71.4 ± 2.1	424.7 ± 2.7	23.8 ± 1.6	>100	>100	36.8 ± 2.4
April	78.7 ± 3.3	204.3 ± 5.2	36.6 ± 4.4	>100	>100	40.3 ± 4.4
May	71.2 ± 2.4	67.4± 4.3	11.7 ± 0.3	37.9 ± 0.3	26.9 ± 0.1	22.0 ± 0.4
June	36.7 ± 2.1	ND	>100	>100	>100	>100
July	65.9 ± 4.1	77.4 ± 2.4	19.4 ± 0.2	>100	>100	30.4 ± 5.9
August	51.8 ± 1.2	384.5 ± 2.6	72.3 ± 1.0	>100	>100	>100
September	79.2 ± 3.8	66.7 ± 2.9	23.5 ± 1.0	79.2 ± 3.3	91.7 ± 6.3	34.1 ± 4.7
October	59.3 ± 3.4	114.7 ± 5.3	35.6 ± 0.3	>100	67.1 ± 8.5	58.4 ± 0.3
November	31.4 ± 1.9	ND	>100	>100	>100	>100
December	63.1 ± 1.7	218.2 ± 3.8	26.2 ± 2.4	86.1 ± 5.2	>100	35.2 ± 4.1
Galanthamine (µM)	87.3 ± 2.1	1.6 ± 0.6	>50	>50	>50	>50
Staurosporine (µM)			0.023 ± 0.002	0.064 ± 0.002	0.175 ± 0.007	0.002 ± 0.000

CEM = T-lymphoblastic leukaemia; MCF7 = breast carcinoma; HeLa = cervical carcinoma; BJ = human fibroblasts
IC_{50} values less than 100 $µg^{-1}$ ml are considered potent inhibitors of AChE and of effective cytotoxicity against cell lines

Owing to the rich spectrum of structural and chemically diverse alkaloid compounds produced by members of the Amaryllidaceae family, it is possible that, relative to changes in environmental factors and other ecological parameters on a month to month basis in the plant growing environment, concurrent changes in alkaloid quality may have resulted, hence the observed variations in cytotoxic activity. A case in point to substantiate this phenomenon, is the homolycorine-type alkaloid content reported in *Lapiedra martinezii* populations growing in different geographic locations which ranged from traces to 91% in the leaf samples and 7 to 62% in bulbs respectively (**RÍOS et al., 2013**). To further emphasise the significant influence of plant growth cycles, developmental stages and tissue differentiation on secondary metabolite quality, the authors reported deoxylycorenine in the bulbs and 6-*O*-methyllycorenine in the leaves as the dominant alkaloids in the species at the vegetative growth stage. A trend of decreasing lycorine-type and increasing homolycorine-type alkaloids was recorded in the bulbs at flowering stage when plants had shed their leaves. However, in addition, an interactive effect between the phenological phases of vegetation dynamics and the spatial heterogeneity between different geographic locations (environmental factors) was demonstrated in some growing areas (those with siliceous soil), where lycorine was the main alkaloid in both leaves and bulbs during vegetative growth (**RÍOS et al., 2013**). Several other field-based ecological studies have demonstrated that vegetation phenology and the associated chemical constituents tends to follow relatively well-defined temporal patterns and that terrestrial ecosystems reflect the Earth's climatic and hydrologic regimes (**LIETH, 1974; WHITE et al., 1997; SCHWARTZ , 1999; CHAGONDA et al., 2000; ZHANG et al., 2003**). Similarly, variations in pharmacological properties from evaluation of seasonally collected plant extracts have mirrored this phenomenon in other related studies (**DERITA et al., 2009; NCUBE, et al., 2011**). The results of the present study could thus be interpreted as a reflection of this complex and multiple interactive effects of numerous factors affecting the quality and quantity of bioactive metabolites.

Although the observed cytotoxic effects of extracts recorded in this study, cannot however, be attributed solely to alkaloids as the plant produces a suite of chemically diverse compounds, it suffice to assume, at least in part that they have had a role, owing to them being toxic. Many of the Amaryllidaceae alkaloids exhibit

antiproliferative properties (**EVIDENTE et al., 2009**) and it has been suggested that these compounds effect this activity by disrupting eukaryotic protein biosynthesis (**HOSHINO, 1998; GRIFFIN et al., 2007**). Some close analogues of these alkaloids, from Amaryllidaceae species, have also been shown to exhibit cytostatic activity and impair cancer cell proliferation and migration by disorganising the actin cytoskeleton (**LEFRANC et al., 2009; VAN GOIETSENOVEN et al., 2010**). Narciprimine, one of the rare, recently isolated alkaloid constituent of *Cyrtanthus contractus* (**NAIR et al., 2011**), has demonstrated selective cytotoxic effect against CEM lymphoblastic leukaemia (IC_{50} 13.3 μM) (**NAIR et al., 2012**). The compound also showed a dose-dependent increase in the proportions of G_2/M phase cells and a reduction in the proportions of G_0/G_1 and S cells. The chemotherapeutic properties of Amaryllidaceae alkaloids is thought to reside mostly with the phenanthridones of the lycorine series (**NAIR and VAN STADEN, 2013**). Lycorine was amongst the first of these compounds to exhibit such activity as demonstrated by its inhibitory effects towards cell division and cell elongation (**COOK and LOUDON, 1952; NAGAKAWA et al., 1956**), as well as protein synthesis in eukaryotic cells (**GRIFFIN et al., 2007**). However, using human glioblastoma cells, **LAMORAL-THEYS et al.** (**2009**) argue that lycorine exerts its *in vitro* activity through cytostatic rather than cytotoxic effects. Lycorine-induced apoptosis was not observed in these cells even at a concentration 10-fold higher than its *in vitro* IC_{50} growth-inhibitory value (**LAMORAL-THEYS et al., 2009**). In their conclusion, the authors suggested that the cytostatic effects induced by lycorine in cancer cells occur by means of an increase in actin cytoskeleton rigidity. Although cytotoxic activities are found across all series of alkaloids of the Amaryllidaceae, **LAMORAL-THEYS et al.** (**2010**) and **NAIR and VAN STADEN** (**2012**) highlight that crinane and lycorine series of compounds are consistently the most active, and remain the most popular targets for such studies with the most potential for clinical development.

By way of comparison, apart from the extracts obtained in May and November (IC_{50} > 100 μg ml^{-1}), most of the extracts with effective cytotoxic effects against cancer cell lines (IC_{50} < 100 $\mu g^{-1}ml$) exhibited selective cytotoxic effects against some cell lines. However, with the exception of the extracts collected in February and August, the rest of these were also cytotoxic against normal human fibroblast (BJ) cells which is an undesirable aspect. The cytotoxicity effects demonstrated by the crude bulb

extracts of *C. contractus* at low concentrations (<100 µg ml^{-1}) in this study suggest their lucrative potential as anticancer agents. The ability of some extracts obtained during certain months of the year to display selective inhibition of specific cancer cell lines points to the general variability in the quality and quantity of bioactive compounds produced across a broad spectra of environmental regimes. Considering that most of the extracts were toxic to normal human fibroblast (BJ) cells, they should thus be viewed and used cautiously.

4.1.3.3. Enzyme inhibition

The results for AChE inhibitory activity are presented in **Table 4.2**. Ten out of the twelve monthly *C. contractus* bulb extracts showed inhibitory activity above 50%. Extracts gathered in March, April, May and September showed good AChE inhibitory activity (> 70%) while those collected in January, February, July, August, October and December exhibiting moderate activity (between 50% and 70%), and the extracts from the remaining two months had low activity (< 40%). However, on determining the IC_{50} values for the dose-dependent inhibitory acitivitity, the two extracts with percentage inhibitory activity < 50% (June and November) did not show a dose dependant activity, and their IC_{50} values were not calculated. Among the extracts which demonstrated potent activity (IC_{50} values < 100 μg^{-1} ml) the hierarchical order of potency in terms of month of collection was: September > May > July > January (**Table 4.2**).

By comparison, based on the IC_{50} values, the four extracts that showed good potency against AChE (January, May, July and September) also demonstrated good cytotoxicity against cancer cell lines in the Calcein AM assay (**Table 4.2**), an attribute that suggest the possibility of similar mechanisms of action by perhaps similar group(s) of compound(s) in these extracts. However, by contrast, when looking at the pattern of alkaloid content during these months (**Figure 4.2**), significant variations exist between all of them although among them, the months of January and September had higher alkaloid content. Furthermore, a snap look at the parameters of weather characteristics in these months (**Table 4.1**) reveals marked differences in terms of the mean rainfall and temperature values and their distribution patterns. Despite the observed monthly variations in weather characteristics and

alkaloid accumulation patterns, the convergence of pharmacological properties displayed by these extracts further alludes to the commonly shared reasoning that quality of the extracts determines biological activity and that, although this attribute varies as a function of season, the fine balance of specific metabolites in the suite of compounds produced in each extract is key to bioactivity. In light of the present results, it is logical to attribute the coincided good monthly pharmacological activities to have been influenced in a similar manner.

When narciprimine, isolated from *C. contractus*, was evaluated *in vitro* for its AChE inhibitory property (**NAIR et al., 2011**) using the standard method of **ELLMAN et al.,** (**1961**) against the known AChE inhibitor galanthamine, the compound demonstrated an appreciable level of dose-dependent activity, with an IC_{50} value of 78.9 µM. As with cytotoxicity, AChE inhibition by Amaryllidaceae plant extracts is attributed to the alkaloid constituents (**ELGORASHI et al., 2004; HOUGHTON et al., 2006; MUKHERJEE et al., 2007**). The Amaryllidaceae alkaloid galanthamine was recently approved in many European countries for the treatment of AD (**HOUGHTON et al., 2006**). The long-acting, selective, reversible, and competitive AChE inhibitory properties of galanthamine has stimulated tremendous research interests for AChE inhibitors from the family Amaryllidaceae (**MUKHERJEE et al., 2007; NAIR and VAN STADEN, 2012**). Significant AChE inhibitory activity for Amaryllidaceae alkaloids other than the galanthamine-type alkaloids such as the lycorine-types assoanine, oxoassoanine and 1-*O*-acetyllycorine has also been reported (**LÓPEZ et al., 2002; ELGORASHI et al., 2004**). Amaryllidaceae alkaloids are classified into different ring types such as crinine-, lycorine-, galanthamine-, cherylline- and tazettine-type alkaloids (**ELGORASHI et al., 2006**). In a quantitative structure-activity relationship study of 24 Amaryllidaceae alkaloids, belonging to five ring types, for AChE inhibition using physicochemical properties as descriptors, **ELGORASHI et al.** (**2006**) demonstrated that substituents at both the aromatic ring and ring C play important roles in the inhibition of this enzyme. With regard to the potent AChE inhibitory activity by some *C. contractus* bulb extracts reported in this study, multiple action/mechanisms by a pool of different types of alkaloids in these extracts can be assumed.

Inhibition of AChE serves as an important strategy for the treatment of AD, Parkinson's disease, senile dementia, myasthenia gravis and ataxia (**BRENNER, 2000**). The good inhibitory activity demonstrated by the extracts in this study adds to the potential list of natural plant-derived sources of AChE inhibitors. Of particular importance, in a developing world context, is the use of these crude extracts in minute quantities in traditional medicine to alleviate neurodegenerative disorders. Although central nervous system (CNS) disorders are not a major concern for the healthcare sector of developing nations, attributable to a number of factors including genetic, dietary, lifestyle and environmental factors entrenched in these societies (**STAFFORD et al., 2008**), 'by contrast, **NAIR and VAN STADEN** (**2013**) highlights the impact of neuronal diseases on western medicine as staggering, affecting one in twenty people over 65 and one in five over the age of 80'.

The percentage inhibition of COX-1 and COX-2 enzymes by *C. contractus* bulb extracts is presented in **Figure 4.3**. The COX inhibitory activity was defined at four levels, with activity below 20% being considered insignificant, 20-40% low, 40-70% moderate, and 70-100% high (**TUNÓN et al., 1995**). Moderate to high levels were considered to be good activity in this study. Although the inhibitory activity of extracts against COX-1 and COX-2 enzymes varied markedly from month to month, the activity fluctuated between moderate to high levels. Of the extracts that showed high inhibitory activity against both enzymes (August, September and December), those obtained in September exhibited the highest COX-1 inhibitory activity, with an inhibition of over 80%. This high COX enzyme inhibitory activity of the extracts obtained in September also coincides with the best AChE inhibition, potent cytotoxic effect (**Table 4.2**) against all cancer cell lines tested in this study and the highest alkaloid content (**Figure 4.3**) within the duration of the study period. Based on these results, it is thus logical to attribute these activities, in part, to the high alkaloid content contained in the extracts.

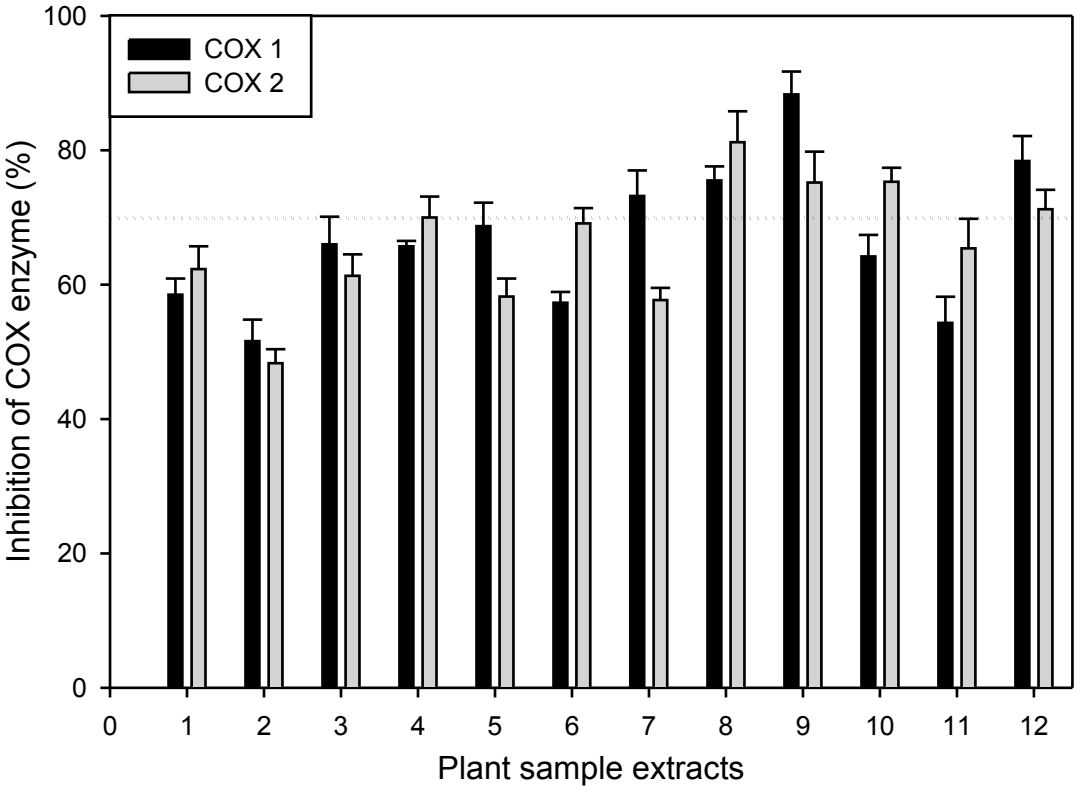


Figure 4.3. Percentage inhibition of COX-1 and COX-2 enzymes by *Cyrtanthus contractus* ethanol bulb extracts collected in (**1**) January (**2**) February (**3**) March (**4**) April (**5**) May (**6**) June (**7**) July (**8**) August (**9**) September (**10**) October (**11**) November (**12**) December. Percentage inhibition by indomethacin (5 µM) was 62.3 ± 2.63 for COX-1 and 64.8 ± 1.93 for COX-2 at 200 µM final concentration

Inflammation is a complex pathophysiological process that involves an interaction of a variety of signalling molecules and mediators in a series of enzyme-catalysed reactions (**WHITE, 1999**). Plant extracts exert their enzyme inhibitory effects through a spectrum of different modes of action and target sites (**CAPONE et al., 2007**). COX-1 enzyme is expressed constitutively in most tissues and catalyses the production of prostaglandins involved in the prostanoid-mediated physiological functions such as gastric cytoprotection, maintenance of renal homeostasis, and normal platelet functions (**MORITA, 2002**). The inducible COX-2 enzyme is thought to be responsible for the accumulation of prostaglandins in most acute inflammations (**VANE et al., 1998**). Anti-inflammatory agents with selective COX-2 inhibition are most desirable as they attenuate the damaging side effects associated with the complete inhibition of COX-1. However, based on the results of this study, all the

tested extracts demonstrated moderate to high inhibition against both enzymes. The extracts such as those with a relatively wider difference between inhibition percentage of COX-1 and COX-2, with COX-2 inhibition being on the higher side (June, October and November extracts, for example), would likely be preferred for use to circumvent the manifestation of the damaging side effects. The development of remedies with specific COX-2 activity remains a considerable challenge.

4.1.3.4. Antimicrobial activity

Although not as impressive as AChE inhibitory activity and cytotoxic effects, a few of the monthly extracts of *C. contractus* bulbs showed good antibacterial activity against some specific strains, with MIC values of 0.78 mg ml^{-1} in each case (**Table 4.3**). In this study, antibacterial MIC values less than, or equal to 1 mg^{-1} ml were considered as good activity (**ALIGIANNIS et al., 2001**). The extracts exhibited a broad spectrum of activity, with MIC values ranging from 0.78 to over 6.25 mg^{-1} ml both between and within monthly extracts. Of the four tested bacterial strains, at least one extract demonstrated good inhibitory activity against at least one strain. The extracts obtained in May and July were the only extracts that showed good activity against at least two bacterial strains, with the July extracts inhibiting three strains. On the other hand, regarding the anticandidal activity, none of the evaluated extracts exhibited good activity against the yeast-like fungus *C. albicans*, although variations in MIC were observed within plant extracts. According to **ZACCHINO et al. (2003)**, an ideal antifungal agent should be composed of compound(s) that are fungicidal with a selective mechanism of action. The lack of fungicidal activity (MFC > 1 mg^{-1} ml) from the tested extracts could be due to lack or low concentration of active compound(s) in these extracts (**NACIF DE ABREU and MAZZAFERA, 2005**).

Table 4.3. Antibacterial and anticandidal activity of seasonally collected *Cyrtanthus contractus* bulb extracts.

Plant extract	Antibacterial activity (MIC mg ml^{-1})				Anticandidal activity (mg ml^{-1})	
	B.s	E.c	K.p	S.a	MIC	MFC
January	3.13	1.56	3.13	6.25	3.13	6.25
February	3.13	3.13	3.13	6.25	6.25	6.25
March	1.56	**0.78**	1.56	3.13	1.56	3.13
April	3.13	6.25	3.13	6.25	3.13	6.25
May	**0.78**	**0.78**	1.56	1.56	1.56	1.56
June	3.13	3.13	6.25	6.25	3.13	3.13
July	**0.78**	1.56	**0.78**	**0.78**	3.13	6.25
August	1.56	1.56	**0.78**	1.56	1.56	1.56
September	3.13	**0.78**	1.56	1.56	1.56	3.13
October	3.13	1.56	1.56	3.13	1.56	3.13
November	1.56	**0.78**	1.56	1.56	6.25	>6.25
December	3.13	3.13	3.13	3.13	3.13	6.25
Neomycin (µg^{-1} ml)	1.95	0.49	0.98	1.95		
Amphotericin B (µg^{-1} ml)					9.77	78.1

Bs = *Bacillus subtilis*, Ec = *Escherichia coli*, Kp = *Klebsiella pneumoniae*, Sa = *Staphylococcus aureus.*
MIC values in bold are considered to be of good activity (MIC < 1 mg ml^{-1})

The good activity shown by some extracts against the Gram-negative bacteria (*E. coli* and *K. pneumoniae*) is of particular interest considering that these bacteria are less susceptible to antibiotics than Gram-positive bacteria (**PAZ et al., 1995; CHARIANDY et al., 1999**) owing to them having lipopolysaccharides on the outer membrane which presents a barrier to various antimicrobial molecules (**SLEIGH and TIMBURY, 1998**). The two Gram-negative bacterial strains are known causative agents for diseases such as, urinary tract and wound infections, bacteriaemia, pneumonia septicaemia and meningitis in humans (**SLEIGH and TIMBURY, 1998**). Of the extracts that exhibited good antibacterial activity, most of them (four out of a total of six), had good activity against *E. coli*. The good activity demonstrated by these extracts against the tested two Gram-negative bacterial strains indicate their

potential value in the treatment of infections caused by these pathogens in traditional medicine. These results are consistent with previous findings on the ethanol extracts of other *Cyrtanthus* species against the same pathogenic strains (**ELGORASHI and VAN STADEN, 2004**).

4.2. Pharmacological evaluation of *in vitro* precursor-fed *Cyrtanthus guthrieae* extracts

4.2.1. Introduction

The recognition of the biological properties of a myriad natural products produced by plants has fuelled the current research focus towards the use of plants as models in the search for new drugs, antibiotics, insecticides, and herbicides. However, this has been made possible by the uncovering of the various and often complex metabolic pathways involved in the biosynthetic processes of these chemical compounds. Plant cell, tissue and organ cultures are identified as promising potential alternative sources for the production of high-value secondary metabolites of industrial and pharmaceutical importance (**RAMACHANDRA RAO and RAVISHANKAR, 2002**). The fact that primary and secondary metabolites cannot be readily distinguished on the basis of precursor molecules, chemical structures and biosynthetic origins, demonstrates the complex inter-linked relationship between the two types of metabolism in plants. Accordingly, some end products of primary metabolism serve as or are shared with secondary metabolic pathways as biosynthetic precursor molecules. For example, all terpenoids, including both primary metabolites and more than 25,000 secondary compounds, are derived from the five-carbon precursor isopentenyl diphosphate, the more than 12 000 known alkaloids are biosynthesised principally from a limited pool of amino acids, with over 8000 polyphenolic compounds being produced by either the shikimic acid pathway or the malonate/acetate pathway (**CROTEAU et al., 2000**). Based on this understanding, coupled with the established pharmacological roles of secondary metabolites, a constant supply of precursor molecules through precursor feeding experiments have, on numerous occasions, yielded desired outcomes (**RAMACHANDRA RAO and RAVISHANKAR, 2002; MULABAGAL and TSAY, 2004**).

As an experimental system, plant tissue culture provides several advantages over the use of outdoor grown plants in that the aspect of seasonality on plant availability is circumvented and the undifferentiated, relatively uniform state of development of cells, tissues and plantlets produced in culture allow for ease of manipulation of various growth factors. Plant cell cultures can synthesise large amounts of secondary products within a relatively short cultivation period. This is very favourable compared with the conventional plant production systems, for which the time frame for alkaloid accumulation for example, may vary from one season for annual plants to several years for some perennial species. The rate of alkaloid biosynthesis can be increased and its study and commercial application is greatly facilitated in culture. Moreover, the greater metabolic rates associated with cell cultures can promote the incorporation of precursors for improved biosynthesis of alkaloid and other secondary compounds. As a medicinally important plant species, *Cyrtanthus guthrieae* cultures evaluated for alkaloid and polyphenolic accumulation patterns in response to precursor feeding (**Section 3.3.2.1**) and the extract samples were evaluated for their pharmacological properties.

4.2.2. Materials and methods

4.2.2.1. Extract preparation and pharmacological evaluation

Plant samples from each of the treatments in the precursor feeding experiment (**Section 3.3.2.1**) were extracted following the procedure described in **Section 4.1.2.1**. The resulting extracts were evaluated for antibacterial, anticandidal, AChE and COX enzymes inhibition, following similar procedures described in **Section 4.1.2** for the respective assays.

4.2.3. Results and discussion

4.2.3.1. Antimicrobial activity

Table 4.4 represents the antibacterial and anticandidal activity of *C. guthrieae* extracts. Although there were variations in the MIC values for both *C. albicans* and

the four tested bacterial strains on a treatment by treatment comparison, none of the extracts exhibited good activity (MIC < 1 mg ml^{-1}). There was no observable link between the levels of the two groups of secondary metabolites (alkaloid and polyphenols) (**Table 3.6**) and the antimicrobial activity obtained in this study. The lack of antimicrobial activity may however, be a reflection that the pool of compounds produced by the plantlets from the precursor-supplemented media does not possess antimicrobial effects against the screened pathogenic strains. One other possible explanation for the lack of activity could perhaps be that the extraction solvent used in this study (ethanol) might have excluded classes of active compounds.

Table 4.4. Antibacterial and anticandidal activity of *Cyrtanthus guthrieae* extracts. Cultures were fed with different concentrations of phenylalanine and tyrosine

Treatment		Antibacterial activity (MIC mg ml^{-1})				Anticandidal activity (mg ml^{-1})	
	µM	B.s	E.c	K.p	S.a	MIC	MFC
Plantlets							
Phe	10	3.13	3.13	6.25	6.25	6.25	> 6.25
	50	3.13	3.13	3.13	3.13	6.25	6.25
	100	3.13	3.13	6.25	3.13	3.13	> 6.25
	1000	1.56	1.56	3.13	1.56	6.25	> 6.25
Tyrosine	10	1.56	3.15	1.56	3.13	3.13	6.25
	50	1.56	3.13	3.15	1.56	> 6.25	> 6.56
	100	1.56	1.56	1.56	1.56	3.13	3.13
	1000	3.13	1.56	1.56	3.13	3.13	3.13
Callus							
Phe	100	1.56	3.56	3.13	1.56	> 6.25	> 6.25
Control	0	3.13	3.13	6.25	6.25	> 6.25	> 6.25

Bs = *Bacillus subtilis*, Ec = *Escherichia coli*, Kp = *Klebsiella pneumoniae*, Sa = *Staphylococcus aureus*; Phe = phenylalanine

4.2.3.2. AChE and COX enzymes inhibitory activity

As reports suggest the most desirable anti-inflammatory agents to have a more selective inhibition activity on COX-2 than COX-1, some of the precursor-fed *C.*

guthrieae extracts in this study exhibited this type of activity (**Table 4.5**). As a general trend, an increase in Phe and tryrosine concentration in the culture media resulted in a progressive increase in the inhibitory activity of COX-2 enzyme by the extracts, with a decrease in inhibition of COX-1. Apart from the low concentration of Phe (10 µM), an increase in both Phe and tyrosine concentration in the medium displayed a similar characteristic trend of increasing inhibition of COX-2 and a decrease in COX-1 inhibition. COX-2 inhibitory activity was also high (> 70%) in all of these treatment extracts (except 50 µM). As pointed out by **MORITA** (**2002**), inhibition of COX-1 may result in the removal of the cytoprotection function, leading to damaging side effects in the gastrointestinal tract. The high inhibitory activity of COX-2 with moderate to relatively low COX-1 inhibition by some extracts in this study presents some interesting insights on the aspect of precursor feeding in *C. guthrieae* cultures for the potential development of selective anti-inflammatory agents. The trend in the results suggest a favoured tilt towards synthesis of compounds active against COX-2 while decreasing the concentration or effects thereof, of those inhibiting COX-1, as the concentration of tyrosine increases in the culture medium. **LUONG et al.** (**1996**) suggest an interaction between the active site chemistry and that of the molecular inhibitor to be one of the factors that determine selectivity. The induced selectivity in the two COX enzymes, as highlighted by **HABEEB et al.** (**2001**), is through interaction of the inhibitor with the secondary pocket of COX-2 which is absent in COX-1. The active compounds in these extracts may be inhibiting COX-2 enzyme through this unique secondary pocket site.

Table 4.5. AChE and COX- (1 and 2) enzyme inhibitory activity of precursor-fed *Cyrtanthus guthrieae* extracts

Treatment		AChE inhibitory activity		COX Inhibitory activity (%)	
Plantlets	µM	(%) Inhibition	IC_{50} (µg ml^{-1})	COX-1	COX-2
Phe	10	73.4 ± 3.1	334.1 ± 4.5	58.4 ± 3.1	62.1 ± 4.8
	50	76.3 ± 2.4	204.6 ± 3.7	63.1 ± 3.4	65.1 ± 2.4
	100	81.2 ± 2.7	**38.9 ± 1.3**	54.3 ± 2.4	73.0 ± 2.8
	1000	83.7 ± 4.2	**74.2 ± 2.1**	46.4 ± 4.1	76.8 ± 3.1
Tyrosine	10	75.6 ± 2.0	388.7 ± 3.3	55.4 ± 2.5	71.7 ± 2.3
	50	85.5 ± 3.0	**80.4 ± 3.5**	40.5 ± 2.8	73.0 ± 3.3
	100	85.9 ± 3.7	**24 1± 1.9**	37.2 ± 4.0	77.4 ± 3.2
	1000	72.4 ± 2.8	**36.0 ± 3.6**	36.9 ± 2.5	80.1 ± 2.6
Callus					
Phe	100	69.3 ± 4.1	246.6 ± 5.2	64.3 ± 4.2	67.3 ± 5.0
Control	0	58.1 ± 36.4	611.7 ± 4.3	52.8 ± 2.2	56.4 ± 3.5

Percentage inhibition by indomethacin (5 µM) was 62.3 ± 2.63 for COX-1 and 64.8 ± 1.93 for COX-2 at 200 µM final concentration
Extracts with IC_{50} values in bold are considered potent inhibitors of AChE

The possibility of the involvement of alkaloids in this activity cannot be ruled out considering that a characteristic increase in alkaloid content was observed (partially up to 50 µM but with significantly high level even at higher concentrations) with the increase in tyrosine concentration (**Table 3.6**). To add further to the potential of *C. guthrieae* precursor feeding in the development of enzyme inhibitors, coincidentally, excellent AChE inhibition activity (IC_{50} value ranges of 24.1-80.4 µg ml^{-1}) was also recorded within the same extracts that displayed high and selective COX-2 inhibition in this study. According to the cholinergic functioning, the replacement of acetylcholine which is progressively lost during the progression of AD delays the loss of cognitive ability. AChE inhibitors function by increasing acetylcholine within the synaptic region thereby restoring deficient cholinergic neurotransmission (**FELDER at al., 2000**). The use of these extracts in traditional healthcare systems may be beneficial in managing the adverse effects of conditions such as AD. One of the early symptoms of AD is neuroinflammation, accompanied by the upregulation of

COX-2 enzyme at the inflammation sites (**HOOZESMANS et al., 2006**). The high COX-2 inhibitory activity demonstrated by these extracts could thus possibly serve a significant role in the control of neuroinflammation in the early pathological progression of AD.

4.3. Conclusions

The possibility of taking advantage of the potential of natural variability of environmental factors in shaping the quality and quantity of ecochemical pools of compounds within plants for the benefit of mankind, can, based on the impressive variations in pharmacological properties observed in this study, be proposed for the future effective development of pharmaceutical agents. The diverse structural chemistry and functions of phytogenic compounds produced by a range of metabolic pathways for adaptation offers a unique resource for novel drug development. Due to the multiplicity of chemical compounds produced by plants under different environmental regimens, their interaction results in complex pharmacological properties with limitless potential. The mechanisms of interaction of different compounds may result in some synergistic effects where the presence of one compound potentiate or amplify the biological activity of another. In certain instances, however, the interaction may be antagonistic, where a chemical compound(s) reduces or inhibit the bioactivity of another compound. There is no doubt, however, that the two mechanisms of action may have been at play among the compounds of the extracts screened in this study.

Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is a promising strategy for the treatment of neurological disorders. Potential sources of AChE inhibitors, anticancer and anti-inflammatory agents is certainly provided by the natural floral resource abundance. The excellent bioactivity exhibited by *C. contractus* bulb extracts in certain months of the year in the present study, particularly in May and September, justifies the coinciding of collection and use of these bulbs for treatment of the related ailments in traditional medicine during these time periods. With regard to precursor feeding in *C. guthrieae*, excellent enzyme inhibitory activity, with some degree of COX-2 inhibition selectivity provides

an interesting dimension of possible drug development through manipulation of biosynthetic pathways. However, be that as it may, and notwithstanding the significant role of the environmental and precursor molecules in shaping the quality of the metabolites within extracts, the possibility of upregulation of toxic/poisonous compounds is equally likely. Isolation and structure elucidation of chemical compounds responsible for the observed bioactivity from each extract can help aid in the chemical synthesis with possible developments of effective drug agents.

CHAPTER 5
General conclusions

Plants form the backbone of all life forms on Earth and are an essential resource for human well-being. The inextricable link between plants and humankind has ensued sustenance of life over generations of human existence. Central to this human-plant interaction, is the fundamental principle that plants provide the predominant ingredients for the various facets of human life. Not only do plants provide food and materials essential for human existence, they also contribute ingredients for human medicine. However, owing to the rapid increase in population growth, urbanisation, agriculture, cultural beliefs coupled with the inadequate provision of basic health care systems, particularly in developing communities, extensive exploitation of plant resources from natural habitats has been on the increase recently. In particular, habitat loss, degradation and fragmentation, has seen some plant species being driven to extinction leading to the progressive loss of plant biodiversity and genetic variability. This setting, however, presents frightening ramifications for humanity. Medicinal plants are among the most affected components of ecosystems, not only as a consequence of habitat loss but partly as a result of direct population decimation by human activity in the wild. In this regard, medicinal plants have become, not only a source of medicine but also provide income to many of the resource poor group of populations through trading, an attribute that further subjects these plants to indiscriminate and unsustainable harvesting from the wild. The Amaryllidaceae family, to which the genus *Cyrtanthus* belongs, comprises of numerous species with great ornamental and medicinal importance. Unique in their floral morphological and chemical constituent diversity (alkaloids in particular), the perennial geophytes of the genus *Cyrtanthus*, endemic to southern Africa, is reported to have a significant number of species under conservation threat. This is largely so because of their demands for horticultural and traditional medicinal purposes. Loss of floral diversity not only threatens stabilisation of ecosystems but to a greater extent, the livelihoods of people who rely on them for survival.

The cultivation of *Cyrtanthus* species, though successful in some species, is complicated by a plethora of impeding factors. Seed propagation is characterised by the often erratic germination potential of the papery seeds produced by most

species. Propagation through bulbil sets is also challenged by the limited number of bulblets produced per annum, with some species unable to produce offsets in some seasons. The often too long growth cycles characterising most species in the genus (up to 15 years to flowering for some species) further adds to the seed propagation challenges for this group of plants. Propagation for large scale conservation or cultivation for commercial production through plant tissue culture techniques would therefore, be a desirable option. The present study sought to explore the *in vitro* propagation potential for *C. contractus, C. guthrieae* and *C. obliquus* as well as to investigate their secondary metabolite content under varying culture conditions and in response to changing external environmental conditions on wild growing populations.

In vitro regeneration through twin-scale bulb scale explants established the differences in optimum culture conditions and media requirements for each species. An effective *in vitro* shoot induction protocol was established through direct shoot organogenesis under plant growth regulator (PGR) combination of 4.4 µM BA/1.1 µM NAA for *C. contractus* and *C. guthrieae* and 6.7 µM/2.7 µM NAA for *C. obliquus*. Significantly higher multiple shoot regeneration was also established for *C. guthrieae* via indirect shoot organogenesis from the regenerated callus cultures under a range of micromolar concentrations (ranges 0.1-1.0 µM) of 2,4-D, picloram and in combination with BA (0.01 and 0.05 µM). In order to optimise adventitious shoot proliferation protocols for the three species, different types and concentrations of cytokinins (CKs) were evaluated. Optimal shoot multiplication frequencies were realised in 5.0 µM TDZ for *C. guthrieae*, 10.0 µM TDZ for *C. contractus* and 10.0 µM BA for *C. obliquus*. *In vitro* shoot regeneration via indirect organogenesis for *C. guthrieae* was approximately two-fold higher compared to direct shoot organogenesis and therefore, offers an improved, efficient and rapid propagation protocol for the species. The use of twin-scale bulb explants produced shoots both on the twin-scale surface and from the basal plates mainly in *C. contractus* and *C. guthrieae* explants. Regeneration of adventitious shoots from the surfaces of the twin-scales has more potential for rapid multiple shoot regeneration as opposed to regeneration from predetermined embryonic bud axils on the basal plate. This attribute presents promising prospects for further manipulation of *Cyrtanthus* scale explants for improved shoot regeneration benefits. In all three *Cyrtanthus* species

rooting was successful without any PGR medium supplementation on full- and half-strength MS media, adding to the reduced regeneration costs, labour and time for the development of micropropagation protocols for the species. The critical and essential stage of the micropropagation procedure, acclimatisation, was achieved successfully in the greenhouse after two weeks of *ex vitro* transfer in all three species, with more than 95% survival in each case. This was a significant step to the successful development and establishment of complete and effective micropropagation protocols for the three species.

Plants undoubtedly provide medicinal benefits to humans largely as a result of their inherent ability to provide a limitless pool of chemical compounds with diverse ranges of medicinal properties. Secondary metabolite production is under the remarkable influence of environmental factors. Manipulation of the culture medium with respect to varying levels of salinity and osmotic stresses on the plantlet cultures (*C. cont*ractus and *C. guthrieae*) gave insightful trends in some phytogenic compounds, including polyphenols. Although the responses varied with species, of particular interest is that some significant increase in polyphenolic compounds were recorded in some treatments relative to the controls. The general trend for both species was a characteristic increase in polyphenolic levels with an increase in stress levels, particularly in response to salinity stress. Likewise, manipulation of the C:N ratios in the culture media gave rise to varied responses in growth rates, alkaloid, polyphenols and primary metabolite levels, with some interesting peaks particularly in alkaloid and polyphenolic compounds, suggesting the potential for increased production of these metabolites in controlled settings. The observed general trend was that of an increased level of polyphenols with increasing carbon (sucrose) concentration in the medium and, to some extent, an increase in alkaloid content as N levels were increased in the medium. These trends were partly in agreement with the carbon to nitrogen theory. Precursor feeding experiments with phenylalanine and tyrosine in *C. guthrieae* cultures also led to varied levels of alkaloid and polyphenolic content, with all treatment levels producing higher levels of the two groups of secondary compounds than the control. Manipulation of the biosynthetic precursor molecules, as evidenced by the results obtained in this study, allows for the improved production levels of compounds of specific interests.

Pharmacological evaluation of the monthly collected *C. contractus* bulbs indicated some impressive bioactivities particularly the cytotoxicity effects against human cancer cell lines and enzyme inhibition (AChE and COX) by the extracts collected in certain months of the year. Of notable interest were the cytotoxicity effects, AChE and COX enzymes inhibitory activity of the extracts collected in May and September. Similarly, some extracts from *in vitro* precursor-fed plantlet and callus cultures demonstrated some excellent bioactivity, against COX and AChE enzymes. The results obtained from this study also reflect on the involvement of the environment in the quality of the extracts produced on a month to month basis and further suggest the importance of coinciding collection and use of plant extracts with the best time of the year or month. The good AChE and COX enzyme inhibitory activity by some of these extracts is of significant importance in the treatment of Alzheimer's disease and neuroinflammation. It can therefore be concluded that these extracts represents an important component of traditional medicine.

Taken collectively, the results from this study provide effective optimal micropropagation protocols for the conservation of the three *Cyrtanthus* species. *In vitro* propagation techniques offer significant prospects for rapid propagation and germplasm storage of the endemic and threatened plant species. Further useful insights on the trends of phytochemical accumulation response patterns in plants under different growing environments are provided for potential future application/exploration in drug development and pharmacological applications as well as optimum times of medicinal usage for different plant extracts.

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