MICROPROPAGATION AND SECONDARY METABOLITES OF *SCLEROCARYA BIRREA*

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Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Research Centre for Plant Growth and Development

School of Biological and Conservation Sciences

University of KwaZulu-Natal, Pietermaritzburg

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STUDENT DECLARATION

Thesis title: Micropropagation and secondary metabolites of S*clerocarya birrea*

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

Student's Full Name: MACK MOYO

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

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SUPERVISOR: PROFESSOR J VAN STADEN

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CO-SUPERVISOR: DR JF FINNIE

DECLARATION 1 - PLAGIARISM

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

PUBLICATION 1

MACK MOYO, MANOJ J KULKARNI, JEFFREY F FINNIE, JOHANNES VAN STADEN (2009). After-ripening, light conditions, and cold stratification influence germination of Marula [*Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro] seeds. *HortScience* 4: 119-124.

Contributions: Laboratory experiments were performed by the first author under the supervision of Prof Johannes Van Staden and Dr Jeffrey F Finnie. Dr Manoj G Kulkarni assisted with data analysis.

PUBLICATION 2

MACK MOYO, JEFFREY F FINNIE, JOHANNES VAN STADEN (2009). *In vitro* morphogenesis of organogenic nodules derived from *Sclerocarya birrea* subsp. *caffra* leaf explants. *Plant Cell, Tissue and Organ Culture* 98: 273-280.

Contributions: All laboratory experiments and microscopy work were done by the first author under the supervision of Prof Johannes Van Staden and Dr Jeffrey F Finnie.

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Contributions: All laboratory experiments and scanning electron microscopy work were done by the first author under the guidance of my PhD supervisors, Prof Johannes Van Staden and Dr Jeffrey F Finnie.

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CONFERENCE PUBLICATIONS

DETAILS OF CONTRIBUTION TO CONFERENCES that form part and/or include research presented in this thesis:

1. Fourth World Congress on Medicinal and Aromatic Plants (WOCMAP IV): 9–14 November 2008; Cape Town International Conference Centre, Cape Town, SOUTH AFRICA.

Title of Poster: **M MOYO, MG KULKARNI, JF FINNIE, J VAN STADEN (2008)**. After-ripening, light conditions and cold stratification of marula [*Sclerocarya birrea* (A. Rich) Hochst. subsp. *caffra* (Sond.) Kokwaro] seeds.

2. Thirty-fifth Annual Conference of the South African Association of Botanists (SAAB): 19–22 January 2009; Stellenbosch University, SOUTH AFRICA.

Title of Paper: **M MOYO, JF FINNIE, J VAN STADEN (2009)**. *In vitro* morphogenesis of organogenic nodules derived from *Sclerocarya birrea* subsp. *caffra* leaf explants.

ABSTRACT

Sclerocarya birrea (marula, Anacardiaceae) is a highly-valued indigenous tree in most parts of sub-Saharan Africa because of its medicinal and nutritional properties. The marula tree is adapted to the semi-arid conditions that characterise most parts of sub-Saharan Africa and renders them unsuitable for conventional crop agriculture. The unique nutritional properties of marula and its high tolerance to dry conditions provide opportunities for its development into a plantation crop. On the other hand, the demand for marula plant parts, mainly the bark and roots as medicinal remedies, poses a great threat to wild populations. In the long term, the growing demand of marula products in the food, pharmaceutical and cosmetic industries will not be sustainable from wild populations alone. Plant tissue culture technologies can be useful for *in vitro* manipulation and mass propagation of the plant in the process of domestication and conservation. The aims of the project were to determine the optimum conditions for seed germination, *in vitro* propagation and plant regeneration, and to evaluate the potential bioactivity of secondary metabolites from its renewable plant parts as an alternative option in the conservation of *S. birrea*.

An *ex vitro* seed germination study indicated that after-ripening and cold stratification are critical factors. Cold stratification (5 °C) of marula nuts for 14 days improved germination (65%) as compared to non-stratified nuts (32%). Direct shoot organogenesis was achieved from leaf explants through the induction of nodular meristemoids on Murashige and Skoog (MS) (1962) medium and woody plant medium (WPM) supplemented with 6-benzyladenine (BA) in combination with naphthalene acetic acid (NAA), indole-3-butryric acid (IBA) and indole-3-acetic acid (IAA). Induction of nodular meristemoids from 86% of the leaf cultures was achieved on a MS medium with 4.0 µM BA and 1.0 µM NAA. High levels (78–100%) of induction were also achieved on WPM with different concentrations of BA (1.0–4.0 µM) and IBA (1.0–4.0 µM). The highest conversion of nodular meristemoids into shoots on MS initiation medium was only 22% for 4.0 µM BA and 1.0 µM NAA. This was improved to 62% when nodular clusters were cultured in MS liquid medium. Histological studies

revealed high numbers of unipolar meristematic buds developing from globular nodules. These embryo-like structures have in the past been mistaken for true somatic embryos. The initiation of high numbers of nodular meristemoids per explant provides potential for automated large-scale clonal propagation in bioreactors, *in vitro* phytochemical production and the development of synthetic seed technology, similar to somatic embryogenesis. Plant regeneration through nodule culture has potential for application in mass micropropagation and plant breeding of *S. birrea*.

Adventitious shoot and root induction are important phases in micropropagation. Plant growth regulators play an important role in these developmental processes, and the type and concentration used have major influences on the eventual organogenic pathway. Three auxins (IAA, IBA and NAA) and four aromatic cytokinins (6-benzyladenine, *meta*-topolin, *meta*-topolin riboside, and *meta*-methoxytopolin riboside) were evaluated for their potential to induce adventitious shoot and root formation in *S. birrea* shoots, hypocotyls and epicotyls. Among the evaluated cytokinins, the highest adventitious shoot induction (62%) was achieved on MS medium supplemented with *meta*-topolin (8.0 µM). The lowest adventitious shoot induction (2.5%) was obtained on MS basal medium containing 2.0 µM *meta*-methoxytopolin riboside. The highest adventitious shoot induction for hypocotyls was 55% on MS medium supplemented with 8.0 µM *meta*-topolin. For the tested auxins, IBA induced adventitious rooting in 91% of shoots at a concentration of 4.0 µM after 8 weeks in culture. However, the *in vitro* rooted plants only survived for two weeks when transferred *ex vitro*. A temperature of 25 °C and 16-h photoperiod were optimum for adventitious root induction. Stomatal density (number per mm^2) on the abaxial leaf surfaces was higher for the 16-h photoperiod treatment (206.6 ± 15.28) compared to that for a 24-h photoperiod (134.6 ± 12.98). Normal mature stomata with kidney-shaped guard cells and an outer ledge over the stomatal pore were observed for *in vitro* plants growing under a 16-h photoperiod.

Total phenolic content, proanthocyanidins, gallotannins, flavonoids, and antioxidant activities of *S. birrea* methanolic extracts were evaluated using *in vitro* bioassays.

Methanolic extracts of the young stem bark and leaves contained high levels of these phytochemicals. *Sclerocarya birrea* young stem extracts contained the highest levels of total phenolics (14.15 ± 0.03 mg GAE g^{-1}), flavonoids (1219.39 ± 16.62 µg CE g^{-1}) and gallotannins (246.12 ± 3.76 µg GAE g^{-1}). *Sclerocarya birrea* leaf extracts had the highest concentration of proanthocyanidins (1.25%). The EC_{50} values of the extracts in the DPPH free radical scavenging assay ranged from 5.028 to 6.921 µg ml^{-1}, compared to ascorbic acid (6.868 µg ml^{-1}). A dose-dependent linear curve was obtained for all extracts in the ferric-reducing power assay. All the extracts exhibited high antioxidant activity comparable to butylated hydroxytoluene based on the rate of *β*-carotene bleaching (89.6 to 93.9%). *Sclerocarya birrea* provides a source of secondary metabolites which have potent antioxidant properties and may be beneficial to the health of consumers.

Sclerocarya birrea young stem and leaf ethanolic extracts exhibited high bioactivity (MIC < 1.0 mg ml^{-1}) against both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria. The highest activity (MIC = 0.098 mg ml^{-1} and total activity = 1609.1 ml g^{-1}) was recorded for young stem extracts against *B. subtilis*. The highest activity (MIC = 1.56 mg ml^{-1} and MFC = 1.56 mg ml^{-1}) in the antifungal assay against *Candida albicans* was observed for young stem ethanolic extracts. *Sclerocarya birrea* extracts had moderate acetylcholinesterase (AChE) inhibition activity. The dichloromethane (DCM) and methanol (MeOH) fractions exhibited dose-dependent acetylcholinesterase inhibitory activity. The highest AChE inhibitory activities were from leaves (DCM fraction, IC_{50} = 0.1053 mg ml^{-1}) and young stems (MeOH fraction, IC_{50} = 0.478 mg ml^{-1}). High inhibitory activity against cyclooxygenase (COX-1 and COX-2) enzymes was observed. All extracts and fractions showed high COX-1 enzyme inhibition (90.7-100%). Petroleum ether (PE) and dichloromethane fractions also exhibited high inhibition against COX-2 enzyme (77.7-92.6%). The pharmacological activities observed suggest that *S. birrea* renewable plant parts (leaves and young stems) provide a substantial source of medicinal secondary metabolites. Based on these results, plant part substitution can be a practical conservation strategy for this species.

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BA	6-Benzyladenine
BHT	Butylated hydroxytoulene
CO_2	Carbon dioxide
COX	Cyclooxygenase
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DPPH	2,2-Diphenyl-1-picryl hydrazyl
EC	Effective concentration
EtOH	Ethanol
GA_3	Gibberellic acid
GAE	Gallic acid equivalents
HCl	Hydrochloric acid
HIR	High irradiance reaction
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC	Inhibitory concentration
INT	Iodonitrotetrazolium chloride
KOH	Potassium hydroxide
*mem*TR	*meta*-methoxytopolin riboside
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MS	Murashige and Skoog (1962) basal medium
*m*T	*meta*-topolin
*m*TR	*meta*-topolin riboside
NAA	α-Naphthalene acetic acid
ORR	Oxidation rate ratio
PE	Petroleum ether
PEM	Pro-embryogenic masses
P_{fr}	Phytochrome far-red
PGR	Plant growth regulator
PPFD	Photosynthetic Photon Flux Density
P_r	Phytochrome red
PVP	Polyvinylpyrrolidone
RPM	Revolutions per minute
SEM	Scanning electron microscope
WPM	Woody plant medium

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Mankind has benefited from plants as sources of nutrition and medicine throughout human existence. Over many generations some elite genotypes of important wild plants have been systematically selected and brought into cultivation to meet the needs of an ever growing population. Despite advances in plant sciences, some valuable wild plants are still utilised as medicinal and food sources from their natural habitats. However, the increasing human population and further expansion of land for agriculture continue to reduce these natural habitats leading to extinction of some plant species, and threatening the existence of numerous others. This threat to plant biodiversity has triggered research in plant conservation, in an effort to curb the growing challenge of plant extinction. South Africa is endowed with an enormous and unique plant biodiversity, which includes high value medicinal, aromatic, herbal, and wild fruits. These plants are utilised by communities contributing significantly to the local economies. Like marula, most of these plants are exploited from the wild and there have been limited efforts to domesticate them.

1.1 DISTRIBUTION AND UTILISATION

Sclerocarya birrea (A. Rich) Hochst. subsp. *caffra* (Sond) Kokwaro (Anacaridiaceae), is an indigenous, drought tolerant multipurpose tree widely distributed in sub-Saharan Africa, between 16 degrees north and 20 degrees south. Its distribution extends from the lowlands of KwaZulu-Natal (South Africa) through tropical Africa into Ethiopia and Sudan **(VILJOEN et al. 2008)**. The sub-Saharan African region is prone to drought, which reduces the yield of staple grains, hence edible wild indigenous plants like marula become an important alternative food source, providing vitamins, minerals, amino acids and trace elements in the diets of the rural population **(MOJEREMANE and TSHWENYANE 2004)**. The Anacardiaceae consists of 73 genera and 600 species of mainly tropical and sub-tropical deciduous trees, shrubs and woody vines **(VILJOEN et al. 2008)**. Other members of the Anacardiaceae, for example, mango (*Mangifera indica*), cashew (*Anacardium*

occidentale), pistachio (*Pistacia vera*) and sumac (*Rhus coriaria*) are of economic importance, both as medicinal and food plants **(VON TEICHMAN et al. 1986)**. An Anacardiaceae species, such as *Rhus glabra* is traditionally used by native Indians of North American in the treatment of bacterial diseases, such as syphilis, gonorrhoea and dysentery **(NASAR-ABBAS and HALKMAN 2004)**. *Spondias mombin* (Anacardiaceae) is medicinally important in the traditional health care systems of the West Indies, Southern Mexico, Peru, Brazil, Equatorial Guinea, Côte D'Ivoire, Nigeria and Sierra Leone **(AYOKA et al. 2006)**. Another species, *Harpephyllum caffrum* is an important medicinal plant in South Africa where the roots, bark and leaves are used in the treatment of several ailments, for example, pain, inflammation, fever, headaches and dysentery **(BUWA and VAN STADEN 2006)**.

1.1.1 FRUIT YIELD

The size and quality of fruits vary considerably from tree to tree, as mass can range from 10 g to more than 60 g **(BOTELLE et al. 2002; SHACKELTON 2003)**. Mean fruit yield per tree was significantly higher for village trees (> 17 000 fruits) than protected area trees (< 3 500), even after accounting for differences in tree size **(SHACKELTON et al. 2003)**. In a fruit yield study conducted by **BOTELLE et al. (2002)** in Namibia, the average production per tree per season was 596 ± 465 kg. The high standard deviation indicated wide variability in fruit yield between trees. Similar work by **LEAKEY et al. (2002)** in South Africa and Namibia indicated significant and continuous variation in fruit mass within each study site. The fruits of 'Namibian Wonder' were larger (69.9 g) than those of other trees assessed in the study **(LEAKEY et al. 2002)**. Furthermore, **BOTELLE et al. (2002)** showed that there was a significant positive correlation (0.67) between canopy size and fruit yield for the Namibian trees.

1.1.2 VITAMIN C CONTENT

The fruits, juice, nuts and oil of marula possess unique properties. Except in South Africa from where the marula-based liqueur (Amarula cream) is marketed internationally, there has been limited commercialisation of the marula products, despite the properties of the fruits and kernels **(SHACKELTON et al. 2003)**.

Considerable research has been conducted on the properties of *S. birrea* fruits, juice, nuts and oil. Marula fruit and juice is rich in vitamin C, providing about 2 mg of vitamin C per g of fresh juice, approximately four to five times the level of average orange juice **(JAENICKE and THIONG'O 2000; MOJEREMANE and TSHWENYANE 2004)**. Studies conducted have reported concentrations of vitamin C ranging from 54–194 mg per 100 g of fruit flesh or juice. The energy value of the fruit is estimated at 130 kJ per 100 g of fruit fresh **(SHACKELTON et al. 2003)**. Hence, the marula fresh fruit is nutritionally important for the rural communities in which the plant grows. Cashew fruits, *Anacardium occidentale*, also have vitamin C levels averaging 200 mg 100 g^{-1} of juice, four times higher than that of orange juice **(TREVISAN et al. 2006)**.

1.1.3 OIL AND PROTEIN CONTENTS

Marula kernels, which comprise of only 10% of the nut, have a high protein and oil content, estimated at 28–31%, and 56–61% respectively **(SHACKELTON et al. 2003)**. Analyses indicate that marula kernels have a higher protein and oil content than most other popular nuts, including walnuts, hazelnuts, chestnuts and almonds **(WYNBERG et al. 2003)**. Fatty acids found in the oil include palmitic acid (12 g 100 g^{-1} fatty acid), stearic acid (9.2 g 100 g^{-1} fatty acid), oleic acid (69.9 g 100 g^{-1} fatty acid), and linoleic acid (7.8 g 100 g^{-1} fatty acid). The energy value of the kernel is approximately 2,699–2,703 kJ per 100 g, which is generally higher than most other commonly consumed nuts except for walnut **(SHACKELTON et al. 2003; WYNBERG et al. 2003)**. Though susceptible to hydrolytic rancidity, marula oil has exceptional resistance to oxidative rancidity, providing high potential for commercialisation. The marula kernels have high levels of magnesium (467 mg 100 g^{-1}), phosphorous (836 mg 100 g^{-1}) and potassium (677 mg 100 g^{-1}) which contributes to the importance of the nuts in the diets of local communities **(WYNBERG et al. 2003)**. Other important nutritional components include mineral elements (iron, calcium, copper, zinc) and vitamins such as thiamine and nicotinic acid **(WYNBERG et al. 2003)**.

1.2 MORPHOLOGY

The genus *Sclerocarya*, named for its hard seed (*Sclero* = hard and *carya* = nut) has four species of which *S. birrea* is the most widely distributed **(SHACKLETON et al. 2003)**. Three subspecies of *S. birrea* are recognised: *S. birrea* subsp. *caffra*, *S. birrea* subsp. *multifoliolata* and *S. birrea* subsp. *birrea*. Subspecies *caffra*, which is the focus of this study is the most widespread and is found in east tropical Africa (Kenya and Tanzania), the Sahelian region, south tropical Africa (Angola, Botswana, Malawi, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe) and Madagascar **(SHACKLETON et al. 2003)**. The tree is common in low to medium altitude areas where rainfall ranges between 200-1600 mm per annum. *S. birrea* is also salt tolerant, thereby extending its distribution into harsh environments and providing a valuable source of livelihood for local communities.

Sclerocarya birrea is a dioecious deciduous tree with an erect trunk and rounded crown. Though predominantly dioecious, some hermaphrodite trees bearing flowers of both sexes can be found. Flowering occurs at the end of the dry season just before the leaves appear and fruits mature at the beginning of the rainy season. Thus, in southern Africa, flowering occurs in September-December, and fruiting from January-June. Female marula trees bear plum-sized stone fruits with a thick yellow peel and a translucent, white, highly aromatic, sweet-sour flesh which is eaten fresh, or used to prepare juices and alcoholic beverages **(NERD and MIZRAHI 1993)**. Fruit abscission occurs before maturation, whilst the fruits are still green and firm. Final ripening takes place on the ground. The marula fruit is a round drupe with a diameter of up to 35 mm. The hard endocarp (stone) is 20-30 mm long and has one to four seed cavities. The seeds are small and fragile and are covered with a thin seed coat.

1.3 POTENTIAL FOR PLANT BIOTECHNOLOGY APPLICATIONS

The immense potential of *S. birrea* is underpinned by its diverse indigenous uses, including nutritional and medicinal purposes, high vitamin C levels (2.0 mg per g of fresh juice), high protein content (60%), high quality stable oil (56% of the kernel) and the novel flavour of its fruit. The attributes of the marula tree provide opportunities for the development of commercial products for the pharmaceutical, cosmetic and food industries.

The potential for commercial development has triggered international domestication research, including germplasm collections and selections of superior phenotypes **(JAENICKE and THIONG'O 2000)**. According to **VON TEICHMAN and ROBBERTSE (1986); NGHITOOLWA et al. (2003);** and **MOLLEL and GOYVAERTS (2004)**, the marula tree has been earmarked for domestication and crop development and improvement due to the commercial potential of its numerous products, the economic role it plays in rural areas and the high possibilities for rural job creation. Compared to other indigenous fruit trees in South Africa, marula has received the most attention in terms of domestication and commercialisation **(VILJOEN et al. 2008)**. In the quest for domestication and commercialisation of the marula plant, plant biotechnologies are likely to provide the best options for plant improvement. Genetic improvement of tree species continues to rely on conventional plant breeding techniques and clonal selections of elite genotypes, which is a slow process **(MULWA and BHALLA 2006)**. For the successful use of plant tissue culture technologies for crop improvement, an efficient and reproducible regeneration procedure is a pre-requisite **(ANATHAKRISHNAN et al. 2002)**. In the case of tree species such as *S. birrea,* the long regeneration cycle and the heterozygous nature of the plants make conventional breeding slow and difficult **(MOLLEL and GOYVAERTS 2004)**. A deeper understanding of the *in vitro* culture manipulation required for its optimum response will generate knowledge that will form the basis for genetic transformation research in plant improvement programmes. Furthermore, plant tissue culture technology provides a practical alternative in the conservation of plant germplasm, especially for important plants.

1.4 PLANT TISSUE CULTURE TECHNIQUES

Success in microculture depends on the intricate and often complex interactions of several factors. Microculture involves the aseptic manipulation of plant tissues which grow under heterotrophic conditions on agar-based media. The type of basal medium and the concentration as well as the balance of nutrients within it, are some of the major factors influencing growth of plantlets *in vitro*. Other factors that influence plant development *in vitro* include the decontamination regime, explants used, plant growth regulators (Sections 1.7 and 1.8), and environmental conditions (e.g. temperature and light) (Section 1.9).

1.4.1 TYPES OF BASAL MEDIA

Lack of a defined nutrient solution capable of inducing sustained growth of plant cells and tissues hindered progress in plant tissue culture during the early 20th century **(VASIL 2008)**. A significant improvement came with the development of White's medium, which included glycine, nicotinic acid, thiamine and pyridoxine **(VASIL 2008)**. The formulation of **MURASHIGE and SKOOG (MS) medium (1962)** was based on White's medium, but included high concentrations of ammonium, nitrate, phosphate and potassium salts as well as chelated iron and myo-inositol **(VASIL 2008)**. **MURASHIGE and SKOOG (MS) medium (1962)** has since become the most commonly used formulation in plant tissue cultures **(BEYL 2005)**, and has formed the basis for subsequent formulations. Other basal medium types have been developed to suit the specific requirements of high salt-sensitive plant species, notably Gamborg B5 medium, Nitsch's medium and woody plant medium (WPM) **(BEYL 2005)**. Of particular interest is the WPM developed by **LLOYD and McCOWN (1981)** for the culture of woody plants. A major difference from the high salt-MS medium is low concentrations of NH_4^+ and NO_3^- ions in WPM (Table 1.1). The concentrations of NH_4^+ and NO_3^- ions in MS are 4.12- and 4.59-fold higher than the levels in WPM.

1.4.2 STERILISATION AND SURFACE DECONTAMINATION

Plant tissue culture media provide ideal conditions for the growth and proliferation of bacteria and fungi, which retard development of cultured plant cells. To achieve aseptic conditions, the growth medium, culture vessels, tools and instruments should be sterilised by autoclaving at 121 °C at a pressure of 15 psi for 20 min **(BEYL 2005)**. The surface decontamination of explants before their introduction into *in vitro* culture is a prerequisite in tissue culture **(TENG et al. 2002)**. Explants should be surface decontaminated using 70% alcohol and sodium or calcium hypochlorite, but various other chemicals can also be used as decontaminants. However, explants which have a vascular system are normally susceptible to internal contamination rendering surface disinfection procedures ineffective **(TENG et al. 2002)**. Such internal infection can be minimised by using antibiotics **(TENG et al. 2002)**.

1.4.3 EXPLANTS

Based on the Cell Theory of Schleiden (1838) and Schwann (1839) **(VASIL 2008)**, and the concept of cellular totipotency **(VASIL and VASIL 1972; RIBNICKY et al. 2002)**, the basic explant for the regeneration of whole plants is a single cell. Thus, plant cell and tissue cultures have the ability to regenerate whole plants from individual cells or groups of highly specialised cells **(VASIL and VASIL 1972)**. This characteristic has formed the basis for plant cell, tissue and organ culture. Different explants can be used for the initiation of plant cultures *in vitro*, and the response can vary both within and between species. Probably the most widely used explant in plant regeneration is the shoot tip due to its meristematic tissues and the inherent ability to minimise contamination. Several authors have reported on the use of different explants for different species with varying degrees of success. For example, shoot tips, nodal stem segments **(KALLAK et al. 1997)**; leaves, internodes, petioles **(BHAU and WAKHLU 2001)**; hypocotyls **(CHOI et al. 2003)**; leaves, petioles, stipules, roots **(PASSEY et al. 2003)**; petals **(MAHAMMADI-DEHCHESHMEH et al. 2008)**, and softwood shoots **(MANSOURI and PREECE 2009)**. Explants that have been used in the *in vitro* culture of Anacardiaceae species are shown in Table 1.2.

1.5 CHALLENGES TO PLANT BIOTECHNOLOGY OF WOODY SPECIES

The application of plant biotechnology, in particular plant tissue culture to woody species, is normally beset by numerous challenges due to their inherent physiology. Some of the major problems in plant tissue culture of woody species include *in vitro* recalcitrance of plant tissues, and phenolic exudation coupled with media and explant browning **(KRISHNA and SINGH 2007)**. These factors are intertwined and their interactions are not fully understood.

TABLE 1.1 Compositions of Murashige and Skoog and Woody Plant Medium

COMPOUNDS	MURASHIGE AND SKOOG	WOODY PLANT MEDIUM
Macronutrients (mg l^{-1}; mM)		
NH_4NO_3	1650 (20.6)	400 (5.0)
$CaCl_2 \cdot 2H_2O$	332.2 (2.3)	96 (0.7)
$Ca(NO_3)_2 \cdot 4H_2O$	-	556 (2.4)
$MgSO_4 \cdot 7H_2O$	370 (1.5)	370 (1.5)
KNO_3	1900 (18.8)	-
K_2SO_4	-	990
KH_2PO_4	170 (1.3)	170 (1.3)
Micronutrients (mg l^{-1}; (µM)		
H_3BO_3	6.2 (100)	6.2 (100)
$CoCl_2 \cdot 6H_2O$	0.025 (0.1)	-
$CuSO_4 \cdot 5H_2O$	0.025 (0.1)	0.25 (1.0)
Na_2EDTA	37.3 (100)	37.3 (100)
$FeSO_4 \cdot 7H_2O$	27.8 (100)	27.8 (100)
$MnSO_4 \cdot H_2O$	16.9 (100)	22.3 (132)
KI	0.83 (5.0)	-
$Na_2MoO_4 \cdot 2H_2O$	0.25 (1.0)	0.25 (1.0)
$ZnSO_4 \cdot 7H_2O$	8.6 (30)	8.6 (30)
Organics (mg l^{-1}; µM)		
Myo-inositol	100 (550)	100 (550)
Glycine	2.0 (26.6)	2.0 (26.6)
Nicotinic acid	0.5 (4.1)	0.5 (4.1)
Pyridoxine HCl	0.5 (2.4)	0.5 (2.4)
Thiamine HCl	0.1 (0.3)	1.0 (3.0)

Adapted from **BEYL (2005)**

1.5.1 *IN VITRO* RECALCITRANCE OF PLANT TISSUES

Recalcitrance occurs when plant cells, tissues or organs do not respond to *in vitro* manipulations that would otherwise induce organogenesis or somatic embryogenesis, and is a major hindrance in plant biotechnological applications **(BENSON 2000a)**. In particular, woody plants are notorious for being highly recalcitrant to *in vitro* treatments. Several authors **(BOGGETTI et al. 1999; DAS et al. 1999; ANANTHAKRISHMAN et al. 2002)** have reported the *in vitro* recalcitrant

nature of an Anacardiaceae species, *Anacardium occidentale* (cashew). **DAS et al. (1999)** further reported that limited success had been achieved in the micropropagation of *A. occidentale* even using seedling explants due to recalcitrance. A myriad of factors influence or trigger recalcitrant responses, namely, whole plant physiology of the donor, *in vitro* manipulations and *in vitro* plant stress physiology **(BENSON 2000a)**. Due to the effects of the physiology of the donor plants some species are amenable to *in vitro* regeneration, whereas others are more difficult **(BENSON 2000a)**. To minimise *in vitro* recalcitrance effects of plant tissues thorough knowledge of the donor plant's life cycle, for example, reproduction phases, rejuvenation and dormancy, is necessary **(McCOWN 2000)**. Correct manipulation of the *in vitro* environment can help overcome the problem of recalcitrant plant tissues. This can be achieved by having an optimum balance of exogenous auxins and cytokinins in the growth medium, and the use of potent cytokinins such as thidiazuron **(BENSON 2000a)**. The application of compounds that are not strictly plant growth regulators, such as polyamines and antioxidants have also been suggested as a means of mitigating recalcitrance in plant tissue culture **(BENSON 2000a)**. *In vitro* stress physiology can induce recalcitrance, and ethylene production and accumulation in culture vessels has been shown to decrease cell proliferation **(KUMAR et al. 1998)**. Another physiological stress factor responsible for early *in vitro* recalcitrance is the oxidation of explants at the time of excision, especially in woody species with high levels of phenolic compounds **(BENSON 2000a)**.

The successful application of plant biotechnology requires the establishment of stabilised cultures to allow for further manipulations in clonal micropropagation and genetic engineering. However, the microculture of woody species is normally problematic because of the inherent episodic growth pattern that characterises most perennial plants **(VIEITEZ et al. 2009)**. Episodic shoot growth involves rapid flushes of growth in the growing season followed by bud dormancy due to predetermined developmental cues that are physiologically not well understood **(McCOWN 2000)**. The episodic shoot growth pattern becomes a major recalcitrance factor in the microculture of woody species because of the inability to achieve fully stabilised shoot cultures which exhibit uniform and continuous growth when sub-cultured **(VIEITEZ et al. 2009)**. In the micropropagation of *Gmelina arborea*, **NAIK et al.**

(2003) observed a steady decline in both shoot and root proliferation over successive sub-cultures. Though the authors attributed the decline in proliferation rates to the effect of repeated exposure to certain levels of cytokinins, the most probable cause was the episodic growth habit of the donor plant and the inability to attain stabilised shoot cultures. Similarly, **NORTON and NORTON (1986)** working on six Rosaceae sp. observed an initial increase in shoot proliferation during the first three generations followed by a systematic decline over the next six sub-cultures. **NORTON and NORTON (1986)** attributed the gradual progressive decrease in shoot proliferation either to epigenetic factors or the episodic shoot growth pattern of the woody species, since the decline coincided with the natural dormant phase. According to **McCOWN (2000)** the episodic shoot growth habit can be overcome by the continuous generation of *de novo* adventitious shoots, or the rejuvenation of *in vitro* cultured plants. **VIEITEZ et al. (1994)** using force-flushed rejuvenated shoots did not observe a decline in shoot proliferation over a two-year sub-culturing period suggesting that the cultures were in the stabilisation phase. In the clonal micropropagation of some *Quercus* sp., **VIEITEZ et al. (2009)** overcame episodic shoot growth by sub-culturing on cytokinin-containing medium on a fortnightly basis over a 6-week period.

1.5.2 PHENOLIC EXUDATION, MEDIA AND EXPLANT BROWNING

Browning of excised explants and the resultant discolouration of culture media is a major challenge in plant tissue culture systems **(HUANG et al. 2002; KRISHNA and SINGH 2007)**. The problem of browning is particularly common in *in vitro* culture of woody species **(POUDYAL et al. 2008)**. **MOLLEL and GOYVAERTS (2004)** reported the prevalence of medium and explant browning of *S. birrea* cultures, and postulated that phenolic compounds produced during wounding were involved in the process. The browning experienced in plant tissue culture is caused by oxidation of phenolic compounds that are released into the medium upon excision of explants. When phenolic compounds are liberated from vacuoles due to excision of explants, they are released into the cytoplasm where oxidation by polyphenoloxidases (PPO) occurs resulting in the formation of coloured quinines **(ROBARDS et al. 1999)**. Browning of excised plant tissues has been reported for several species, for example, *Phyllostachys nigra* **(HAUNG et al. 2002)**, *Oryza sativa* **(LI et al. 2007)**,

Plantanus occidentalis **(TAO et al. 2007)**, *Musa acuminata* **(KO et al. 2009)**, *Pinus syvestris* **(PIRTTILÄ et al. 2008)**. The optimum pH of 5.8 used in plant tissue culture is conducive for PPO the optimum pH range of which is 5.0-7.0 **(ROBARDS et al. 1999)**. The effects of phenolic exudation and the coupled browning of plant tissues and medium is normally minimised by the use of antioxidants (e.g. ascorbic acid) or adsorptive materials (e.g. polyvinylpyrrolidone, activated charcoal). Response to anti-browning treatments varies depending on several factors, such as plant species, genotype and time of explanting. In *P. nigra* neither ascorbic acid nor polyvinylpyrroridone curbed the problem of browning **(HAUNG et al. 2002)**. Significant control of browning in *Pyrus* sp. was achieved using either ascorbic acid or polyvinylpyrroridone **(POUDYAL et al. 2008)**. Advances in plant genetic engineering are providing more options in understanding the molecular mechanisms involved in browning of plant tissues. Recently, **LI et al. (2007)** reported that *Oryza sativa* callus browning was controlled by a single chromosomal locus (*Induced callus 1*; *Ic1*). An understanding of the molecular mechanisms may help to explain the physiology of browning and provide effective anti-browning strategies in the future.

1.6 MICROPROPAGATION OF ANACARDIACEAE

The Anacardiaceae family comprises some commercially important plant species of mainly tropical and subtropical deciduous trees. Some members of the Anacardiaceae that are of commercial value and are now cultivated, include, *Pistacia vera* (pistachio), *Mangifera indica* (mango) and *Anacardium occidentale* (cashew). Attempts in plant biotechnological applications have mainly focused on these Anacardiaceae species (Table 1.2). There is a conspicuous lack of literature on the *in vitro* propagation of *S. birrea*.

1.6.1 *PISTACIA VERA*

Pistachio (*Pistacia vera*) is a drought tolerant multipurpose tree species which is economically important for local farmers in the arid and semi-arid areas of Iran, Turkey, Algeria and other regions **(BENMAHIOUL et al. 2009)**. The expansion of pistachio in these regions has been limited by inefficient propagation methods and unsatisfactory pollination arising from non-fully synchronised flowering of male and female plants **(TILKAT et al. 2009)**. These challenges in pistachio seed production

stimulated research in plant tissue culture and related biotechnology applications. Using embryogenic tissue obtained from kernels of immature fruits, **ONAY et al. (1995)** induced somatic embryos on MS medium supplemented with 4.4 µM BA. **ONAY et al. (1996)** produced encapsulated somatic embryos and there was no difference with or without 1.0 mg l^{-1} BA. **BARGHCHI and ALDERSON (1996)** demonstrated the control of *in vitro* shoot-tip necrosis using calcium (12-24 mM). Multiple shoots (20.4 per explant) were induced from nodal segments of mature trees on MS medium supplemented with 8.8 µM BA. Addition of BA (9.0 µM), GA_3 (0.2 µM) and silver nitrate (24.0 or 48.0 µM) improved regeneration frequency and shoot growth of nodal segments of *in vitro* seedlings **(OZDEN-TOKATLI et al. 2005)**. Silver (Ag^{2+}) ions interact and block ethylene binding sites thereby counteracting ethylene-induced recalcitrance **(OZDEN-TOKATLI et al. 2005)**. Adventitious shoot regeneration was achieved from *in vitro*-derived mature leaf explants supplemented with 1.0 mg l^{-1} IAA and 2.0 mg l^{-1} BA **(TILKAT and ONAY 2009; TILKAT et al. 2009)**, and shoot multiplication was highest on 1.0 mg l^{-1} BA **(TILKAT et al. 2009)**.

1.6.2 *MANGIFERA INDICA*

Mango (*Mangifera indica*) is an important fruit tree in many parts of the tropical regions, and has received scientific attention including application of recent developments in plant biotechnology research. **LITZ et al. (1982)** reported on the induction of somatic embryogenesis from ovular nucellus tissue of polyembryonic mango cultivars using 20% coconut water or 2 mg l^{-1} BA. Using mature leaf tissues and combinations of auxins (IAA, NAA) and cytokinins (BA, kinetin) **RAGHUVANSHI and SRIVASTAVA (1995)** induced both callus and shoot formation, with the highest shoot proliferation on 1.0 µM IAA and 13.0 µM kinetin. **ARA et al. (1999)** encapsulated cotyledonary-stage somatic embryos in 2% calcium-alginate, achieving germination and conversion of 73.6% and 45.8%, respectively. **LI et al. (2008)** using [^{3}H]IAA demonstrated polar auxin transport from the distal to the proximal end of cotyledonary segments. The authors also observed two patterns of root induction depending on the auxin pre-treatment. Adventitious roots (94.7%) were formed on the abaxial surface when cotyledons were pre-treated with 2700 µM NAA, and on the proximal cut surface with pre-treatments of 2900 µM IAA (86.7%), 492.1 µM IBA (85.6%) and 2500 µM IBA (90%) **(LI et al. 2008)**. Most of the work on mango

micropropagation has focused primarily on somatic embryogenesis **(LITZ et al. 1982; JANA et al. 1994; MONSALUD et al. 1995; LAD et al. 1997; ARA et al. 1999; ARA et al. 2000; PATEÑA et al. 2002; RIVERA-DOMÍGUEZ et al. 2004; XIAO et al. 2004; WU et al. 2007)**, probably due to the envisaged advantages for plant biotechnological applications. For example, whereas genetic clones created through shoot organogenesis require further manipulation for root initiation, in somatic embryogenesis complete plants with a bipolar axis, vascular system and functional meristems are produced in a single step **(BASSUNER et al. 2007)**. Somatic embryogenesis also provides for the long-term storage of plant tissues through cryopreservation, a process which stops cellular metabolic activity **(BOMAL and TREMBLAY 2000)**, large-scale clonal micropropagation of elite genotypes and easy manipulation in plant transformation research **(BANDYOPADHYAY and HAMILL 2000)**. The amenability of somatic embryos for long-term storage provides an alternative strategy for plant germplasm conservation. A critical requirement for cryopreservation is the reduction of tissue water content using cryoprotectants, an osmotic pretreatment or dehydration **(BOMAL and TREMBLAY 2000)**.

TABLE 1.2 Micropropagation of some important Anacardiaceae species

SPECIES	EXPLANT	RESPONSE IN CULTURE	REFERENCES
Pistacia vera	Mature leaf	Direct plant regeneration	**TILKAT et al. 2009**
	In vitro-derived mature leaf	Direct shoot organogenesis	**TILKAT and ONAY 2009**
	Embryo axes; axillary buds	Embryo germination; axillary bud proliferation	**BENMAHIOUL et al. 2009**
	Nodal	Shoot growth	**OZDEN-TOKATLI et al. 2005**
	Shoot tips	*In vitro* grafting	**ONAY et al. 2004**
	Nodal	Axillary shoot initiation	**ONAY 2000**
	Somatic embryos	Synthetic seeds	**ONAY et al. 1996**
	Shoot tips	Control of shoot tip necrosis	**BARGHCHI and ALDERSON 1996**
	Shoot tips	Plant regeneration	**DOLCET-SANJUAN and CLAVERIA 1995**
	Immature kernels	Somatic embryogenesis	**ONAY et al. 1995**
	Cotyledons; embryonic axes	Shoot organogenesis	**KITTO and McGRANAHAN 1992**
	Shoot tips	Rhizogenesis	**RUGINI 1992**
	Pollen	Pollen germination	**GOLAN-GOLDHIRSH et al. 1991**
Anacardium occidentale	Nodal segments	Shoot development	**GEMAS and BESSA 2006**
	Seed coat	Direct somatic embryogenesis	**MARTIN 2003**
	Zygotic embryos	Direct somatic embryogenesis	**GOGATE and NADGAUDA 2003**
	Cotyledons	Direct plant regeneration	**ANANTHAKRISHNAN et al. 2002**
	Nucellar tissue	Somatic embryogenesis	**CARDOZA and D'SOUZA 2002**
	Shoot tips; nodal cultures	*In vitro* grafting	**THIMMAPPAIAH et al. 2002a**

SPECIES	EXPLANT	RESPONSE IN CULTURE	REFERENCES
	Shoots	Axillary shoot bud proliferation and elongation; rooting; micrografting	**THIMMAPPAIAH et al. 2002b**
	Shoot tips	*In vitro* micrografting	**MNENEY and MANTELL 2001**
	Microshoots	Rhizogenesis	**BOGGETTI et al. 2001**
	Nucellus	Somatic embryogenesis	**GOGATE and NADGAUDA 2000**
	Shoot nodes	Shoot and root organogenesis	**BOGGETTI et al. 1999**
	Embryos	Direct plant regeneration	**DAS et al. 1999**
	Nucellus	Somatic embryogenesis	**ANANTHAKRISHNAN et al. 1999**
	Shoot tips; leaf axils; cotyledonary nodes	Shoot and root organogenesis	**DAS et al. 1996**
	Cotyledonary nodes; microshoots	Shoot and root organogenesis	**D'SILVA and D'SOUZA 1992**
	Cotyledons	Direct plant organogenesis	**PHILIP 1984**
Mangifera indica	Shoot tips	Control of shoot tip necrosis and browning	**KRISHNA et al. 2008**
	Cotyledonary segments	Adventitious root formation	**LI et al. 2008**
	Immature cotyledons; nucellus	Direct somatic embryogenesis	**WU et al. 2007**
	Cotyledons of immature zygotic embryos	Direct somatic embryogenesis	**XIAO et al. 2004**
	Nucellus from immature fruits	Somatic embryogenesis; plant regeneration	**RIVERA-DOMÍNGUEZ et al. 2004**

SPECIES	EXPLANT	RESPONSE IN CULTURE	REFERENCES
	Pro-embryogenic masses	Genetic transformation (Particle bombardment)	**AGREZ et al. 2004**
	Nucellar tissue	Somatic embryogenesis; plant regeneration	**PATEÑA et al. 2002**
	Protoplasts isolated from PEMs of nucellar tissue	Somatic embryogenesis; plant regeneration	**ARA et al. 2000**
	Nucellar tissue	Encapsulated somatic embryos; germination and plant regeneration	**ARA et al. 1999**
	Nucellar tissue	Somatic embryogenesis	**LAD et al. 1997**
	Mature leaf	Callus; shoot formation	**RAGHUVANSHI and SRIVASTAVA 1995**
	Somatic embryos	Embryogenic suspension culture	**MONSALUD et al. 1995**
	Nucellar tissue	Somatic embryogenesis	**JANA et al. 1994**
	Somatic proembryos	*Agrobacterium tumefaciens*-mediated transformation	**MATHEWS et al. 1992**
	Ovules	Somatic embryogenesis	**LITZ et al. 1982**
Sclerocarya birrea	Shoot tips	*Agrobacterium tumefaciens*-mediated transformation	**MOLLEL and GOYVAERTS 2004**

PEM-Pro-embryogenic mass

1.6.3 *ANACARDIUM OCCIDENTALE*

Cashew (*Anacardium occidentale*) is a fruit tree valued for its edible nuts and cashewnut shell liquid, and has attracted interest in conventional plant breeding and biotechnology programmes with the goal of improving productivity. The kernels are globally in demand in the confectionary industry **(GOGATE and NADGAUDA 2003)**. Growing demand of the kernels has lead to an increase in levels of cultivation of the crop, but the current propagation methods are inadequate in supplying planting material **(GOGATE and NADGAUDA 2003)**. Using cotyledons **PHILIP (1984)** observed shoot organogenesis with supplementation of MS medium with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} kinetin. **D'SILVA and D'SOUZA (1992)** achieved a maximum of 40 multiple buds from cotyledonary nodes using 22.2 µM BA. The buds were elongated on MS medium supplemented with 100 ml l^{-1} coconut water and 4.4 µM BA. In a study by **ANANTHAKRISHNAN et al. (1999)** embryogenic calli were produced with the inclusion of 6.78 µM 2,4-D, and upon transfer to liquid medium supplemented with 4.52 µM 2,4-D somatic embryos were induced. Using nucellar tissue, **CARDOZA and D'SOUZA (2002)** obtained more stable embryogenic calli with picloram (0.5 mg l^{-1} and 1.0 mg l^{-1}) compared to 2,4-D. Proliferation into globular embryos occurred upon transfer to MS medium supplemented with 0.5 mg l^{-1} picloram and 1.0 mg l^{-1} putrescine, before maturation into cotyledonary embryos with 0.5 mg l^{-1} abscisic acid. **GEMAS and BESSA (2006)** evaluated the influence of carbohydrate type and concentration and concluded that a combination of fructose and maltose (each at 83 mM) increased shoot proliferation and elongation in a single step. Table 1.1 provides a summary of advances in the application of plant tissue culture technologies for some commercially important Anacardiaceae species.

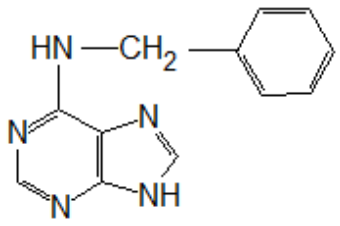
1.7 CYTOKININS: STRUCTURE, METABOLISM AND FUNCTION

Plant growth regulators are low molecular weight natural products that regulate plant physiological and developmental processes at micromolar or lower concentrations **(SKOOG and MILLER 1957; BAJGUZ and PIOTROWSKA 2009)**. Cytokinins together with auxins constitute the most important group of plant growth regulators that control plant growth and development in plant tissue culture systems. Until recently only the isoprenoid cytokinins such as zeatin, isopentenyladenine and dihydrozeatin were solely considered to be naturally occurring in higher plants

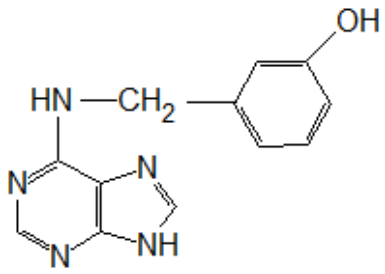
(STRNAD 1997; STRNAD et al. 1997). Zeatin is the most biologically active of the isoprenoid class of cytokinins, and *trans*-zeatin is characterised by higher activity in plant tissue culture compared to its stereoisomer *cis*-zeatin **(BAJGUZ and PIOTROWSKA 2009)**. Recently, **STRNAD et al. (1997)** reported on the occurrence of aromatic N^6-substituted adenine derivatives as another independent class of natural cytokinins in plants. The naturally occurring N^6-substituted adenine cytokinins exist as free compounds, ribosides or glucosides **(GASPAR et al. 1996)**.

1.7.1 METABOLISM OF AROMATIC CYTOKININS

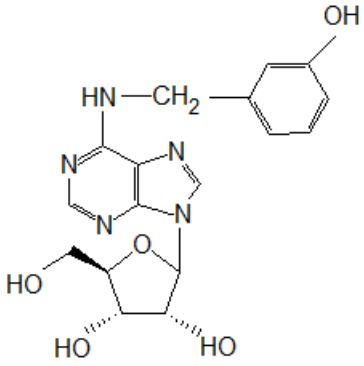
Endogenous aromatic cytokinins are highly active organic compounds comprising N^6-benzyladenine and its derivatives **(BAJGUZ and PIOTROWSKA 2009)**. Hydroxylated derivatives of N^6-benzyladenine in *meta* and *ortho* positions of the benzyl group include 6-(3-hydroxybenzyl)adenine (*meta*-topolin), 6-(2-hydroxybenzyl)adenine (*ortho*-topolin), benzyladenine, and their metabolites **(STRNAD 1997)**. Natural cytokinins occur in plant tissues as free bases, nucleosides and nucleotides, which form reversible or irreversible conjugates with sugars and amino acids **(BAJGUZ and PIOTROWSKA 2009)**. The conjugates act as storage, transport and biologically inert forms of cytokinins **(BAJGUZ and PIOTROWSKA 2009)**, thus providing plants with physiological and developmental plasticity. **VAN STADEN and CROUCH (1996)** suggested that cytokinin bioactivity may be a result of their metabolic products. Exogenous free cytokinin bases that are applied to induce shoot proliferation in plant tissue culture can also be converted into nucleosides and nucleotides, and further transformed into conjugates **(WERBOUCK et al. 1995)**. Fig. 1.1 is an illustration of 6-benzyladenine and some of the *meta*-topolin derivatives used in plant tissue culture.



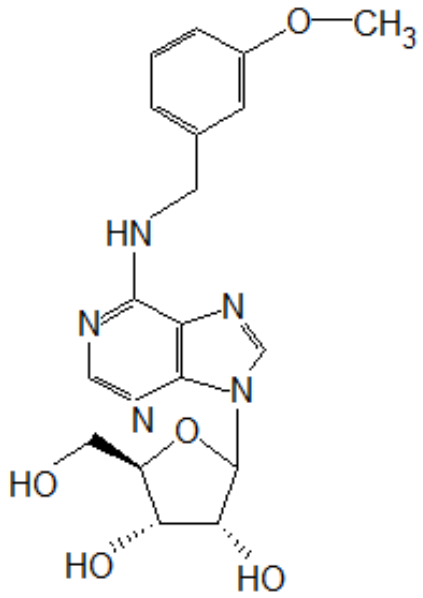
(a) 6-Benzyladenine (BA)



(b) *meta*-Topolin (*m*T)



(c) *meta*-Topolin riboside (*m*TR)



(d) *meta*-Methoxytopolin riboside (*mem*TR)

FIGURE 1.1 Chemical structures of selected aromatic cytokinins.

1.7.1.1 Cytokinin interconversion

Cytokinin metabolism involves their transformation into either metabolically active or inert forms in plant tissues, and includes conversions and interconversions of bases, ribosides, glucosides, side chain modifications, conjugation and conjugate hydrolysis reactions **(SCHMÜLLING 2004)**. Exogenous cytokinins in plant tissue culture systems are converted into a diverse range of metabolites that include products of purine ring substitution (e.g. riboside, nucleotide, *N*-glucoside) or aromatic side chain cleavage (e.g. adenine) **(VAN STADEN and CROUCH 1996)**. A major feature of cytokinin metabolism involves the interconversion of free base, riboside and nucleotide forms **(STRNAD 1997)**, and is a critical mechanism in the regulation of

cytokinin compounds **(SCHMÜLLING 2004)**. The enzymes that catalyse the metabolism of adenine and adenosine may also be involved in interconversion reactions **(STRNAD 1997)**. The riboside class of metabolites constitutes the transport form of cytokinins **(VAN STADEN and CROUCH 1996)**. The authors further stated that the functional or active cytokinin pool consists of the free base, nucleosides and mononucleotides, which are readily interconvertible thereby ensuring a steady supply of precursors. In particular, nucleotides act as storage reservoirs of active forms of cytokinins **(VAN STADEN and CROUCH 1996)**.

1.7.1.2 Cytokinin conjugation

Cytokinin conjugation by glycosylation of the purine ring or side chain into stable or transient forms is a biochemical transformation process that plays a crucial role in the regulation of the active cytokinin pool **(AUER 1997)**. Exogenously applied cytokinins in plant tissue culture may undergo irreversible modifications to form *N*-glucoside and *N*-alanine conjugates resulting in the loss of biological activity **(VAN STADEN and CROUCH 1996; BAJGUZ and PIOTROWSKA 2009)**. Cytokinin N^7- and N^9-glucoside conjugates are stable compounds that cannot be hydrolysed and re-metabolised into the free base form **(AUER 1997)**, and the biological activity of which is considerably lower in relation to the free base forms **(STRNAD 1997)**. N^3-conjugates may be biologically inactive, but can be hydrolysed into active forms **(SCHMÜLLING 2004)** unlike the N^7- and N^9-conjugates. Thus, the only functional exception for the cytokinin-*N*-glucosides might be N^3-glucosides, which have been shown to be biologically active and may represent a storage form of cytokinins **(KAMÍNEK et al. 1997)**.

Cytokinin *O*-glucosides in which glucose is substituted in the N^6-side chain position have not been observed for benzyladenine **(VAN STADEN and CROUCH 1996)**, but constitute the major metabolite form of *meta*- and *ortho*-topolin **(STRNAD 1997)**. Unlike *N*-glucosides, the *O*-glucoside species serve as storage cytokinins and are readily interconvertible to the biologically active free base form **(KAMÍNEK et al. 1997; STRNAD 1997)**. *O*-glucosylation gives rise to storage cytokinin forms which are biologically active in the glucoside form or may yield free bases through the activity of *β*-glucosidases, thus ensuring a prolonged steady release and supply of

active forms in plant tissue culture systems **(WERBROUCK et al. 1996; STRNAD 1997)**. Working on the micropropagation of *Spathiphyllum floribundum*, **WERBROUCK et al. (1995)** observed that *O*-glucosylation in *meta*-topolin was facilitated by the occurrence of a hydroxyl on its benzyl ring, and the resultant product was broken down by *β*-glucosidases. Cytokinin-*O*-glucosides and are localised in the vacuoles **(KAMÍNEK et al. 1997)**.

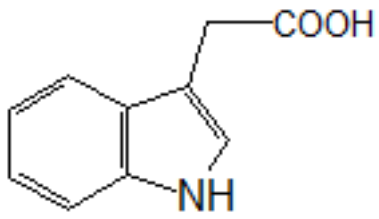
1.7.1.3 Cytokinin activity

Cytokinins play a central signalling role in plant development at the cellular level by regulating the cell cycle and inducing expression of certain genes, and at the organ level by promoting adventitious shoot formation or inhibiting root growth **(WERNER et al. 2001; BAJGUZ and PIOTROWSKA 2009)**. Exogenously applied cytokinins stimulate several other metabolic and developmental processes including chloroplast development, delaying leaf senescence and *in vitro* induction of caulogenesis **(TORELLI et al. 2006)**. Synthetic benzyladenine is the most commonly used and successful cytokinin for the induction of multiple shoots in micropropagation **(VAN STADEN and CROUCH 1996)**. However, attempts to overcome micropropagation challenges such as hyperhydricity and shoot-tip necrosis have stimulated the search for new cytokinins and the recent discovery of the class of plant growth regulators, the *meta*- and *ortho*-topolins.

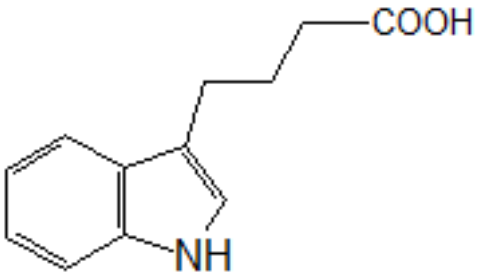
1.8 AUXINS: STRUCTURE, METABOLISM AND FUNCTION

Auxins, the first plant growth regulators to be discovered, constitute a chemically diverse group and are characterised by an aromatic structure such as an indole, phenyl or naphthalene ring with a carboxyl group attached to the side chain **(BAJGUZ and PIOTROWSKA 2009)**. Following the discovery of the naturally occurring indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) were chemically synthesised **(DE KLERK et al. 1999)**. Auxins regulate many physiological processes in plant growth and development, including rhizogenesis and apical dominance. The induction of rhizogenic activity has practical implications in micropropagation as the application of exogenous auxin is a requirement in many plant species **(DE KLERK et al. 1999)**. The most widely used auxin for rooting in commercial micropropagation is indole-3-

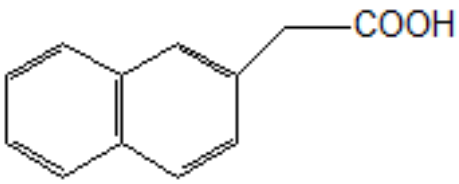
butyric acid **(DE KLERK et al. 1999)** due to its stability in culture. The chemical structures of IAA, IBA and NAA are illustrated in Figure 1.2.



(a) Indole-3-acetic acid (IAA)



(b) Indole-3-butyric acid (IBA)



(c) α-Naphthalene acetic acid (α-NAA)

FIGURE 1.2 Chemical structures of commonly used auxins.

1.8.1 FACTORS AFFECTING AUXIN PHYSIOLOGY

The effectiveness of different auxins in inducing rhizogenesis depends on numerous factors and their interactions, principally; concentration of free auxin reaching target cells, affinity for the auxin receptor proteins, conversion of the exogenous compound, and concentration of endogenous auxin **(DE KLERK et al. 1999)**. Exogenously applied auxin is rapidly taken up by plant cells **(DE KLERK et al. 1999)** through a chemiosmotic diffusion pH gradient **(BLAKESLEE et al. 2005)** and influx/efflux carriers **(MARKS et al. 2002)**. Upon uptake into plant cells auxins may undergo conversion through oxidation and conjugation **(DE KLERK et al. 1999)**. Oxidation of auxins, particularly IAA and IBA (to a lesser extent) leads to an irreversible loss of activity **(EPSTEIN and LUDWIG-MÜLLER 1993)**. Indole-3-acetic acid is five times more prone to photo-oxidation than IBA **(FOGAÇA and FETT-NETO 2005)**. On the other hand α-NAA is not photo-destroyed or oxidised in either the growth medium or in the plant upon uptake **(SMULDERS et al. 1990)**. Unlike the irreversible oxidation reactions, all three auxins, IAA, IBA and NAA can be conjugated to sugar, amino acid or inositol derivatives resulting in reversible inactivation of the auxins **(DE KLERK et al. 1999; FOGAÇA and FETT-NETO 2005)**. Conjugated auxins are hydrolysed yielding biologically active free auxin, and also serve as transport and storage forms **(SMULDERS et al. 1990)**. Conjugation and oxidation of auxins in plant tissue culture reduces the free biologically active form to about 1% or less **(DE KLERK et al. 1999)**.

Auxins in plant tissues are detected by specific glyco-protein receptors to which they reversibly bind, but do not chemically convert as in enzyme systems **(LIBBENGA and MENNES 1995)**. The diversity of responses by plant species to different auxins may be due to selectivity of the auxin-binding protein receptors **(DE KLERK et al. 1999)**. The occupied receptors trigger a signal transduction cascade, which stimulates the rhizogenic response **(LIBBENGA and MENNES 1995)**. Different concentrations of auxins have a significant effect on root formation and proliferation in microculture and higher concentrations of IBA and NAA may have a strong inhibitory effect on rooting **(DE KLERK et al. 1995)**.

In plants, the principal endogenous auxin, IAA, is synthesised in the meristematic tissues **(BLAKESLEE et al. 2005)** and transported from cell to cell in a unidirectional process termed polar auxin transport **(MUDAY and DELONG 2001)**. The polar auxin transport mechanism provides signals for developmental processes such as vascular differentiation, organ development and tropic growth **(BLAKESLEE et al. 2005)**.

1.8.2 AUXINS AND INDUCTION OF RHIZOGENESIS

DE KLERK et al. (1999) distinguished three distinct rooting phases in apple microcuttings *viz*., dedifferentiation, induction and formation of root meristemoids. The dedifferentiation phase occurs within the initial 24 h of exposure to an auxin stimulus, and is characterised by acquisition of competence by cells and accumulation of starch grains. This developmental phase is characterised by molecular and biochemical processes preceding morphological expression **(FOGAÇA and FETT-NETO 2005)**. In the induction phase (72 to 96 h) the now competent cells become committed to root primordium formation **(DE KLERK et al. 1999)**. During this rhizogenic phase starch grains become degraded, cell division is initiated and 30-celled meristemoids become evident **(DE KLERK et al. 1999)**. After the formation of meristemoids the requirement for auxins ceases, and the once-optimal auxin concentrations can become inhibitory **(DE KLERK et al. 1999)**. Meristemoids develop into root primordia and eventually roots during the differentiation **(DE KLERK et al. 1999)** or expression phase **(FOGAÇA and FETT-NETO 2005)**.

1.9 ENVIRONMENTAL CONTROL IN MICROCULTURE

Optimisation of the microenvironment is a key step in micropropagation and ensures the production of good quality plantlets that have high chances of surviving the *ex vitro* conditions in greenhouses and ultimately the natural environment. The culture vessel is a miniature greenhouse or growth chamber with tightly controlled conditions **(KOZAI et al. 1997)**. The characteristics of the conventional *in vitro* environment include; constant temperature, high relative humidity, low photosynthetic photon flux density (PPFD), optimised concentrations of sugars, salts and plant growth regulators in the growth medium, aseptic conditions, but also accumulation of secondary metabolites that may be toxic **(KOZAI et al. 1997)**.

1.9.1 LIGHT

Light, which is the source of energy for photosynthesis, is an important factor for plant growth and development in tissue culture. In particular, light intensity and photoperiod in the microenvironment affect plantlet physiological processes and growth. However, light may be required to a lesser degree in plant tissue culture systems for the regulation of photosynthesis and photomorphogenesis **(ECONOMOU and READ 1987)**. Though the light requirement for photosynthesis of *in vitro* plants is of less importance compared to *in vivo* plants **(ECONOMOU and READ 1987)**, it still profoundly regulates plant development through influences of intensity, photoperiod and spectral quality **(DA SILVA and DEBERGH 1997)**.

1.9.1.1 Photosynthetic photon flux density

Light energy for photosynthesis is generally measured by photosynthetic photon flux density (PPFD) on the growing plantlet **(IBARAKI and NOZAKI 2005)**. In plant tissue culture systems low light intensities (15-65 µmol m^{-2} s^{-1}) are normally used, as exposure to high PPFD can result in photoinhibition and photo-oxidative damage to the fragile photosynthetic apparatus **(KODYM and ZAPATA-ARIAS 1998)**. Similarly, **NGUYEN et al. (1999)** stated that excess light energy above the PPFD saturation point leads to photo-inhibition, that is, depression of the photosynthetic efficiency of the plantlets under *in vitro* conditions. The PPFD saturation point determines the maximum photosynthetic rate, and depends on the CO_2 concentration in the culture vessel **(NGUYEN et al. 1999)**. Increasing light intensity reduces the requirement for light-harvesting complexes (LHC) of *in vitro* plants **(LEE et al. 2007)**. In low light regimes plants acquire high levels of chlorophyll *a/b* binding LHCs especially those associated with photosystem II, whereas in high light conditions plants increase levels of photosystems, ATP synthase and Calvin cycle enzymes, in particular Rubisco **(LEE et al. 2007)**. Low PPFD levels induce production of relatively higher concentrations of chloroplast pigments and electron carriers, and increased numbers of chloroplasts **(WALTERS et al. 2003)**. In the micropropagation of *Withania sonnifera*, **LEE et al. (2007)** recorded the highest growth (e.g. shoot length, root length, leaf area, fresh weight, and dry weight) at a PPFD of 30 µmol m^{-2} s^{-1}. The authors further observed increases in chlorophyll and carotenoid contents and number of stomata up to a PPFD of 60 µmol m^{-2} s^{-1}.

1.9.1.2 Photoperiod

Photoperiod, which is the length of the light period, is one of the critical environmental cues that regulate plant developmental processes such as stem elongation, bud dormancy, leaf growth, flowering **(KÜHN et al. 2009)**, pigment accumulation, hormone synthesis, gene expression and ion influx changes **(HAUSER et al. 1998)**. Plants have a system of sensory photoreceptors that monitor changes in the ambient light environment **(GYULA et al. 2003)**. The photoperiodic signal is also perceived and regulated by photoreceptors, in particular phytochromes (red/far-red receptors), cryptochromes (blue/UV-A light receptors) and phototropins (phot1 and phot2) **(WALKER and BAILEY 1968; RUDIGER et al. 1983; MOCKLER et al. 2003)**. Phytochromes are plant chromoproteins that exist in two photo-interconvertible forms, the red-absorbing (P_r) and the physiologically active far-red absorbing (P_{fr}) molecules **(MOCKLER et al. 2003)**. Phytochromes are synthesised in the inactive red-light-absorbing (P_r) form and undergo photoconversion to the P_{fr} iso-form in a reaction optimised at red wavelengths **(FRANKLIN 2009)**. Cryptochromes are flavoproteins **(GYULA et al. 2003)** that regulate developmental processes in plants such as de-etiolation and flowering **(SULLIVAN and DENG 2003)**. Phototropins, the most recently characterised family of photoreceptors **(BRIGGS and CHRISTIE 2002)** are flavoproteins **(GYULA et al. 2003)** known to influence chloroplast movement and stomatal opening in plants **(GYULA et al. 2003; SULLIVAN and DENG 2003)**.

One advantage of plant tissue culture systems is that the photoperiod can be modified to suit the required conditions. Both rhizogenic and caulogenic processes are regulated by photoperiod under *in vitro* conditions **(ECONOMOU and READ 1987)**. Variable responses may be elicited between plant species and different explants of the same species under similar photoperiodic treatments **(ECONOMOU and READ 1987)**. **KÜHN et al. (2009)** distinguished between natural and artificial photoperiods. Natural photoperiods are characterised by twilight phases, which may constitute a critical part in the daily cycle of plant development **(KÜHN et al. 2009)**. On the other hand, an artificial photoperiod regime lacks the twilight period, as the transition between light and dark phases is abrupt **(KÜHN et al. 2009)**. This major difference in photoperiod dynamics between the artificial conditions in growth

chambers and natural environment may have physiological implications on plants during acclimation.

1.9.1.3 Light quality

Light quality is measured by the spectral wavelength and has significant effects on plant development and morphogenesis *in vitro*. Plants respond differently to the spectral wavelengths of light and the relationships become even more complex due to interactions with other factors such as temperature, photoperiod and light intensity. White light with a wavelength band of 400-700 nm, provided by cool fluorescent lamps is normally used for the maintenance of tissue cultured plants. Other spectral wavelengths of light that have been demonstrated to influence *in vitro* plant morphogenesis are blue (450 nm), red (666 nm) and far-red light (730 nm). As discussed in the previous section, the perception of light signals occurs through a network of specialised photoreceptors.

In embryo cultures of *Pseudotsuga menziesii*, red light enhanced adventitious bud formation, whereas blue light had no effect **(ECONOMOU and READ 1987)**. In *Pelargonium* sp., **APPLEGREN (1991)** observed that red light increased stem elongation, whilst blue light had an inhibitory effect. *In vitro Solanum tuberosum* plants cultured under an 18-h photoperiod in red light were thin, long and had small leaves, whereas those in blue light remained short and thick with well-developed leaves **(AKSENOVA et al. 1994)**. When the photoperiod was shortened to 10 h, plants under blue light increased in length **(AKSENOVA et al. 1994)** attesting to the complex interplay of various factors on plant morphogenesis. **D'ONOFRIO et al. (1998)** observed that red light induced the highest number of somatic embryos in *Cydonia oblonga* compared to other wavelengths of light. The authors concluded that phytochrome and cryptochrome photoreceptors exerted opposite effects on *C. oblonga* somatic embryogenesis. In *Withania somnifera* significant increases in leaf area, fresh weight, dry weight and chlorophyll content under white fluorescent light and a mixture of red and blue light were recorded, compared to monochromatic effects of blue and red lights **(LEE et al. 2007)**. More stomata were found on plants growing under fluorescent light or a mixture of blue and red spectral light **(LEE et al. 2007)**. Similarly, using a mixture of red and blue light, **KIM et al. (2004)** observed increased rates of photosynthesis and growth of *Chrysanthemum* plantlets. These

observations suggest that monochromatic light causes an imbalance in light energy required for the functioning of the photosynthetic apparatus **(GOINS et al. 1997)**. On the other hand, the spectral energy distribution of fluorescent or a mixture of blue and red light coincides with that of chlorophyll absorption **(LEE et al. 2007)**.

1.9.2 TEMPERATURE

Plant morphogenesis is a complex developmental process that is controlled and regulated by the interplay of multiple environmental stimuli, of which temperature is one of the most important **(FRANKLIN 2009)**. The biophysical and biochemical process of photosynthesis is temperature dependent **(GOMES-LARANJO et al. 2006)** and is a major determinant of the rate of growth of plants **(SAMACH and WIGGE 2005)**. In nature the perception of ambient temperature allows for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability **(FRANKLIN 2009)**. However, in culture a constant temperature regime is normally maintained and this may have effects on the development of *in vitro* plants. For example, **NADGAUDA et al. (1997)** attributed the lack of synchrony in *in vitro* anthesis of *Bambusa arundinacea* to the maintenance of constant temperature and light conditions when compared to *in vivo* flowering.

1.10 LIQUID SHAKE CULTURE

Liquid cultures provide an alternative to solid-based media, and have potential for mass multiplication and scaling-up using commercial bioreactors. **PAEK et al. (2005)** describes a bioreactor as a self-contained, sterile environment that is designed for intensive culture of propagules and facilitates the control of the microenvironmental conditions, including aeration, temperature, dissolved oxygen and pH. The potential for *in vitro* mass-production of important plants is partially limited by the high costs caused by intensive manual handling in solid-based media systems **(NHUT et al. 2004)**. Labour costs account for at least 60% of the total production costs in conventional micropropagation **(GUPTA and TIMMIS 2005)**, and the problem can be alleviated by using liquid culture technology due to its amenability to automation. **GUPTA and TIMMIS (2005), PAEK et al. (2005) and ZIV (2005)** suggested that robotic automation of scaled-up liquid cultures in bioreactors via

organogenesis or somatic embryogenesis would reduce costs that normally typify conventional techniques.

1.10.1 FACTORS AFFECTING GROWTH IN LIQUID CULTURES

A major limitation of liquid shake cultures is the development of abnormal shoots exhibiting hyperhydricity symptoms **(CHEN and ZIV 2003; HAHN and PAEK 2005; McALISTER et al. 2005; NHUT et al. 2004; PAEK et al. 2005; WAWROSCH et al. 2005; ZHU et al. 2005; ZIV 2005)**. The excessive accumulation of water in plant cells, associated with hyperhydricity can induce oxidative stress and cause damage to micropropagules due to the production of reactive oxygen species (ROS) **(ZIV 2005)**. The excessive production of ROS under these circumstances leads to recalcitrance and loss of morphogenetic competence **(BENSON 2000b)**. Continuous agitation of cultures in liquid shake medium can also cause shearing damage and the breakdown of cell walls **(ZIV 2005)**.

Despite these potential drawbacks, liquid cultures offer several overarching advantages over solid-gelled cultures: in particular, higher growth rates; optimal supply of nutrients and plant growth regulators allowing for rapid uptake by cells; renewal of the culture atmosphere; changing of medium according to developmental stage; and filtration of medium for secondary metabolites **(GUPTA and TIMMIS 2005; ZIV 2005)**. Solid-based medium culture systems create gradients of nutrient and plant growth regulator concentrations within containers, thereby slowing growth **(GUPTA and TIMMIS 2005; PRASAD and GUPTA 2006)**. In liquid shake cultures, the rapid uptake of nutrients and plant growth regulators creates a highly efficient system in which lesser concentrations are required compared to agar-based medium formulations **(GUPTA and TIMMIS 2005; HAHN and PAEK 2005)**. Liquid culture systems also allow for the dilution of phenolic compounds to levels that are not harmful to *in vitro* plants **(GUPTA and TIMMIS 2005)**. This is a critical factor for woody species characterised by high concentrations of such secondary metabolites.

Other factors affecting growth and proliferation in liquid cultures have to be controlled to facilitate uniform development of micropropagules, namely, the gaseous constituents, nutrient balance, pH, temperature and light **(ILAN et al. 1995)**. Success

of liquid cultures depends to a larger extent on the efficient transfer of oxygen from the gaseous to the liquid phase **(PAEK et al. 2005)**, and the removal of other gases and volatile compounds which may accumulate in the culture vessel **(ILAN et al. 1995)**. The concentration of oxygen in the gaseous phase in liquid cultures can be regulated by varying the degree of agitation and through aeration **(ZIV 2005)**. The rate of agitation creates a critical diffusion gradient which drives oxygen into the aqueous phase. Agitation is necessary because of the low solubility of oxygen in water (0.25 mmol l^{-1} at 25 °C, 1 atmosphere, 21% (v/v) O_2 in the air) **(PAEK et al. 2005)**. The pH in liquid cultures has been reported to fluctuate over the culture period mainly due to the imbalance of ammonium ions **(PAEK et al. 2005)**. The pH in liquid cultures can drop to 4.5 or lower values due to the uptake of ammonium ions, and rise to at least 5.5 with the utilisation of nitrate ions **(ZIV 2005)**. The pH can be stabilised by constantly replenishing the medium.

1.10.2 MORPHOGENESIS IN LIQUID CULTURES

The immense potential for large-scale micropropagation of valuable plants has been highlighted by several authors (**ILAN et al. 1995; GUPTA and TIMMIS 2005; HAHN and PAEK 2005; WAWROSCH et al. 2005; PRASAD and GUPTA 2006)**. Somatic embryogenesis, in which a bipolar structure is produced, provides the preferred morphogenetic pathway for ease of manipulation in liquid cultures. A key advantage of somatic embryogenesis is that both the root and shoot meristems of the bipolar structure are produced in a single step. Somatic embryos are amenable to liquid culture systems because they are produced in large numbers; are relatively small; have a uniform size; and can be maintained at a certain developmental stage and stored for long periods **(PAEK et al. 2005)**. The potential for long-term storage of dehydrated or partially dehydrated somatic embryos through cryopreservation provides flexibility and synchronisation in high-throughput production systems, and also facilitates transportation **(PAEK et al. 2005)**.

On the other hand, the large-scale micropropagation of plants regenerating unipolar structures such as protocorms, buds and shoots offers similar potential and advantages, but has been limited due to the problem of hyperhydricity of leaves and shoots **(ZIV 2005)**. A practical solution to the development of hyperhydric tissue in

liquid cultures is the induction of meristemoid clusters through the use of growth retardants **(ILAN et al. 1995)**. Fortunately, in some plant species the morphogenetic induction of meristemoid clusters or nodules occurs naturally without addition of plant growth retardants, but this advantage has not been harnessed for mass Micropropagation **(ILAN et al. 1995)**. The envisaged mass microproduction potential of nodule cultures offers an alternative micropropagation system **(BATISTA et al. 2000)** to somatic embryogenesis. Nodule clusters are composed of actively dividing and densely-packed meristematic cells, which continuously form new meristemoids **(ZIV 2005)**. The nodular stage can be maintained in the long-term providing for synchronisation and potential for automated micropropagation technology **(TENG 1997)**. The formation of nodules and their inherent potential for large-scale micropropagation have been reported in several plant species, including *Populus* sp. **(McCOWN et al. 1988; FERREIRA et al. 2009)**, *Nerine* sp. **(ZIV et al. 1994)**, *Ananas comosus* **(TENG 1997)**, *Humulus lupus* **(BATISTA et al. 2000)**, *Phalaenopsis* sp. **(YOUNG et al. 2000)**, *Charybdis* sp. **(WAWROSCH et al. 2005)** and *Rosa* sp. **(TIAN et al. 2008)**. A major advantage of meristematic nodules is their low susceptibility to shearing and cell wall breakdown **(ZIV 2005)** due to their compactness **(ZIV et al. 1994)**.

1.11 PLANT SECONDARY METABOLITES AND THEIR BIOACTIVITIES

Plant secondary metabolites constitute a heterogeneous group of compounds that are not necessarily essential as part of the primary biochemical pathways of cell development in plant growth **(MAKKAR et al. 2007)**. Though structurally diverse, these compounds are mainly synthesised from a limited pool of biosynthetic precursors such as phosphoenolpyruvate, pyruvate, acetate and acetyl CoA **(ROBARDS et al. 1999)**. Some of the commonly occurring plant secondary metabolites include phenolic compounds, alkaloids and saponins. Plant secondary metabolites also referred to as phytochemicals possess a range of therapeutic properties, including antibacterial, anti-inflammatory, anti-carcinogenic, antioxidant and acetylcholinesterase inhibitory activities, hence their use in phytomedicine for centuries.

Phenolic compounds are derived from the shikimate and phenylpropanoid pathways, and constitute a diverse and ubiquitous class of plant secondary metabolites characterised by an aromatic ring and one or more hydroxyl groups **(ROBARDS et al. 1999)**. The main groups of phenolic compounds that occur in plants include flavonoids and tannins.

1.11.1 FLAVONOIDS: OCCURRENCE AND BIOLOGICAL ACTIVITY

Flavonoids represent a structurally and chemically diverse class of low molecular weight phenolic compounds that are characterised by the flavan nucleus **(HEIM et al. 2002)**. Their occurrence is widespread as they are constituents of fruits, vegetables, seeds, nuts, leaves, flowers, bark and roots of a diverse range of plants **(COOK and SAMMAN 1996; HEIM et al. 2002)**. More than 4 000 structurally unique flavonoid compounds have been identified in vascular plants **(COOK and SAMMAN 1996; HEIM et al. 2002; MIDDLETON et al. 2008)**. Fig. 1.3 depicts the generic structure of a flavonoid.

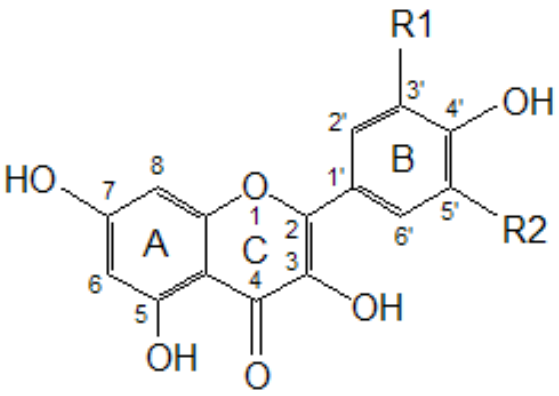


FIGURE 1.3 Generic chemical structure of a flavonoid.

Due to their ubiquitous occurrence in green plant cells and ease of separation through chromatographic techniques, flavonoids are used as reliable markers in taxonomic studies **(HAVSTEEN 2002)**. Flavonoids have important functions in plant biochemistry and physiology. In plants, flavonoids protect cells against ultraviolet radiation, pathogens and herbivores **(HEIM et al. 2002)**, and have recently been shown to modulate the activity of *P*-glycoproteins in polar auxin transport **(MURPHY et al. 2000; PEER and MURPHY 2007)**. Flavonoids are known to regulate several other plant biological mechanisms such as energy transfer, photosynthesis,

respiration and morphogenesis **(MIDDLETON et al. 2008)**. Anthocyanins provide the red, pink, blue, mauve, orange and violet colouration in flowers, fruits, wines and some vegetables **(HEIM et al. 2002)**, and release the stimuli that facilitates pollination by insects (**COOK and SAMMAN 1996**).

The use of plants as medicinal remedies since ancient times is partially attributed to the biological activities of flavonoids. **MIDDLETON et al. (2008)** suggested that the biochemical and pharmacological activities of flavonoids was due to their long association with animal species and other biological organisms. Some of the proven biological activities include antimicrobial, antiviral, anti-inflammatory, antioxidant, antiallergic, vasodilatory effects and inhibition of lipid peroxidation **(COOK and SAMMAN 1996)**. Various types of phenolic compounds isolated from medicinal plants, including flavonoids are effective against potentially antibiotic-resistant bacteria such as *Staphylococcus aureus* **(HATANO et al. 2005)**. The antioxidant activity of flavonoids is recognised by their strong chain-breaking actions, thereby protecting cells against the detrimental effects of ROS **(MIDDLETON et al. 2008)**. ROS is a collective term for oxygen-centred radicals such as superoxide, and hydroxyl, and non-radical oxygen derivatives, namely hydrogen peroxide and singlet oxygen, which can cause oxidative damage to plant proteins, DNA and lipids (**SUZUKI and MITTLER 2006**). However, though ROS can cause oxidative damage to plant cells, recent studies have shown that ROS also act as signal transduction molecules, in physiological processes such as stomatal closure, mitosis and programmed cell death; and play a key role in plant defence mechanisms against pathogen infection **(FOYER and NOCTOR 2005; SUZUKI and MITTLER 2006)**. In humans the overproduction of ROS can also result in tissue injury and has been implicated in disease progression and oxidative damage to nucleic acids and proteins **(MIDDLETON et al. 2008)**. Nonenzymatic antioxidants are classified as either hydrophilic (e.g. ascorbic acid) or lipid-soluble (e.g. carotenoids, vitamin E), depending on whether they primarily act in the aqueous phase or the lipophilic region of the plasma membranes **(MIDDLETON et al. 2008)**.

The inhibitory effects of flavonoids on mammalian enzymes including lipooxygenases and cyclooxygenases have been demonstrated in several *in vitro* systems **(MIDDLETON et al. 2008)**.

1.11.2 TANNINS: OCCURRENCE AND BIOLOGICAL ACTIVITY

Tannins are secondary metabolites that do not constitute a unified chemical group, but are characterised by a wide diversity of chemical structures **(CHAVAN et al. 2001)**. These polyphenolic compounds are broadly categorised into two groups, these are, hydrolysable tannins and condensed tannins (proanthocyanidins) **(MAKKAR et al. 2007)**. Hydrolysable tannins consist of carboxylic acids that are esterified to a carbohydrate molecule such as glucose by either gallic acid (gallotannins) or ellagic acid (ellagotannins) **(OSZMIANSKI et al. 2007)**. Condensed tannins or proanthocyanidins are flavanol-based polymers that are classified into extractable and bound condensed tannins **(MAKKAR et al. 1999)**. The chemical structures of a gallotannin and proanthocyanidin are depicted in Fig. 1.4 and Fig. 1.5, respectively. Tannins occur widely in the plant kingdom and are known to have important ecological, anti-nutritional and medicinal functions **(MAKKAR et al. 1999; OKUDA 2005)**. Some ecological functions of tannins include protection of seeds against insects, birds and herbivore attack **(CHAVAN et al. 2001)**. Bound condensed tannins may have anti-nutritional effects due to their high affinity for proteins, and thus form insoluble complexes with digestive enzymes and dietary proteins **(CHAVAN et al. 2001)**. Both condensed and hydrolysable tannins have been reported to possess various nutritional and medicinal properties such as immunomodulating effects, antibacterial, antifungal, antiviral, free radical scavenging, anti-inflammatory, anti-diarrhoeal and anti-tumour activities **(OKUDA 2006; TOMCZYK and LATTÉ 2009)**. The free radical scavenging activities are thought to reduce health risks associated with cardiovascular diseases and cancer **(OSZMIANSKI et al. 2007)**. **TOMCZYK and LATTÉ (2009)** attributed the anti-diarrhoeal efficacy of condensed tannins to the formation of a protective layer by tannin-mucosal protein complexes.

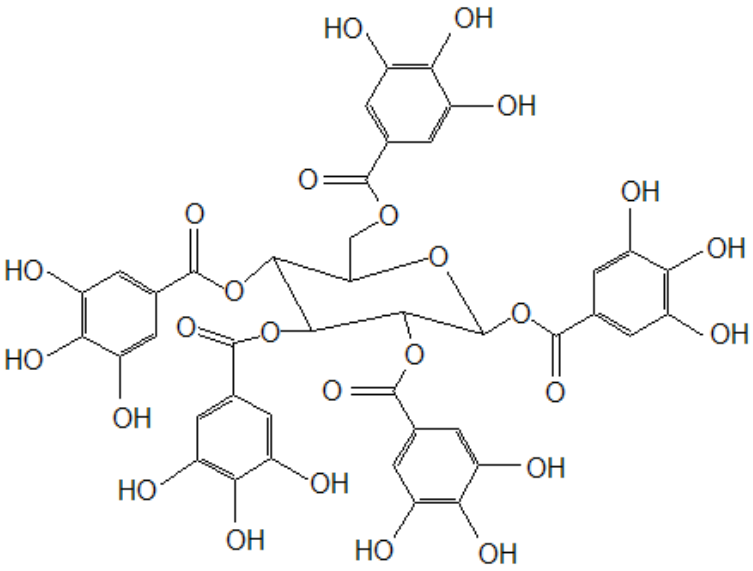


FIGURE 1.4 Chemical structure of a gallotannin (hydrolysable tannin) showing gallic acid molecules esterified to glucose.

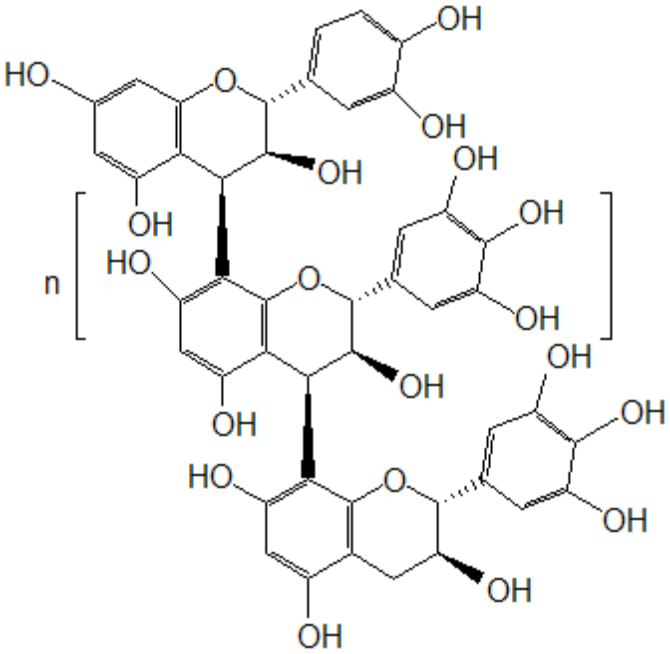


FIGURE 1.5 Chemical structure of a proanthocyanidin depicting the polymeric flavonoid sub-units consisting of epicatechin and catechin.

1.11.3 INFLAMMATION AND ANTI-INFLAMMATORY AGENTS

Cyclooxygenase (COX) is a key enzyme that catalyses the synthesis of prostaglandins from their precursor, arachidonic acid. Prostaglandins are bioactive lipids that mediate physiological and pathophysiological effects, such as

inflammation, pain, fever and homeostasis **(KURUMBAIL et al. 2001)**. Inflammation is a localised response by cells and tissues to injury and infection. Acute inflammation is a rapid and short defensive response against microbial attack, whereas chronic inflammation is characterised by prolonged duration **(IWALEWA et al. 2007).** Two isoforms of cyclooxygenase that are encoded by different genes have been identified, that is, the constitutive COX-1 enzyme and COX-2 enzyme which is induced at inflammation sites *in vivo* **(KURUMBAIL et al. 2001)**. The COX-1 isoform is responsible for housekeeping roles, for example, cytoprotection by maintaining the gastrointestinal epithelium **(KAM and SO 2009)**. Nonsteroid anti-inflammatory drugs (NSAIDS) which are commonly used as anti-inflammatory agents, have been shown to cause undesirable side effects such as irritation, bleeding and gastrointestinal ulcers (**CALATAYUD et al. 2002; BOTTING 2006)**. The mode of action of NSAIDs, for example, aspirin, indomethacin and ibuprofen is through their inhibitory effects on mainly the COX-1 enzyme thereby preventing the pathological synthesis of prostaglandins **(BOTTING 2006)**. The preferential inhibition of COX-1 may be related to the removal of the cytoprotection leading to damaging side effects in the gastrointestinal tract **(GILROY et al. 1998)**.

Due to the problem of side effects of NSAIDs, research has focused on finding selective COX-2 anti-inflammatory agents. Since COX-2 is the predominant isoform present at inflammation sites demonstrating pro-inflammatory effects, its selective inhibition was likely to minimise development of gastrointestinal ulcers and related side effects **(CALATAYUD et al. 2002).** However, although selective COX-2 inhibitors have been demonstrated to significantly reduce the problem of gastro-toxicity, they increased the likelihood of cardiovascular disorders **(BOTTING 2006)**. Parallel to the search for anti-inflammatory medicines, has been the exploration of natural products exhibiting comparatively effective efficacy. The baseline in the search for natural anti-inflammatory agents has been informed by the reported utilisation of plants in folk medicine against conditions such as pain, inflammation and fever. Traditional medicines provide a vast resource base for the potential discovery of new drugs, which may have minimal side effects **(KÜPELI and YESILADA 2007)**. In this context, several researchers have reported the anti-inflammatory activities of flavonoids and other secondary metabolites. Examples of the anti-inflammatory activities of phenolic compounds, predominantly flavonoids from medicinal plants are

given by several authors, including **RAO et al. (2005)**, **CLAVIN et al. (2007)**, **KÜPELI and YESILADA (2007)** and **AQUILA et al. (2009)**.

1.11.4 ACETYLCHOLINESTERASE INHIBITION

In the pathological progression of Alzheimer's disease (AD), neuroinflammatory symptoms are coupled with the upregulation of COX-2 at the inflammation sites **(HOOZESMANS et al. 2006)**. The upregulation of COX-2 provides a potential target for therapeutic intervention **(HOOZESMANS et al. 2006)**. Research evidence using biochemical markers suggests that inflammation may be a target in the early stages of the disease **(WEGGEN et al. 2007)**. Alzheimer's disease is an age-related neurological disorder that is characterised by progressive loss of cognitive ability and behavioural abnormalities. It is a major problem in developed countries where the population of old people is higher. The cholinergic hypothesis, which associates the role of acetylcholine in neurotransmission, provides the most widely accepted mechanism regulating AD **(FELDER et al. 2000),** and has become the basis for its treatment. Acetylcholinesterase (AChE) is the key enzyme in the hydrolysis of the neurotransmitter, acetylcholine in the nervous system, into choline and acetic acid **(MUKHERJEE et al. 2007)**. Inhibition of AChE restores the level of acetylcholine necessary for memory processing and storage in elderly people. Besides synthetic medicines, plants are potential sources of AChE inhibitors **(MUKHERJEE et al. 2007)**. Already, galanthamine a natural pharmaceutical product which was isolated from an Amaryllidaceae species is widely used **(HEINRICH and TEOH 2004)**. To date a variety of plants have shown promising bioactivity in AChE inhibition, and efforts abound in isolating and characterising the active phytoconstituents **(MUKHERJEE et al. 2007)**.

1.12 SIGNIFICANCE AND AIMS OF THE STUDY

The conservation of plant species is important for the maintenance of plant biodiversity. Finding alternative plant regeneration methods that are efficient remains one of the key conservation strategies. Plant tissue culture technology provides the means for the mass propagation of elite genotypes and the application of downstream biotechnological tools, such as molecular breeding. In the case of medicinal plants where the bark and roots are mainly preferred, evaluating the

pharmacological bioactivity of renewable plant parts may provide for non-destructive and sustainable harvesting. The aims of the study were to develop a micropropagation system for *S. birrea* partially using histological techniques to explore *in vitro* plant development; and to evaluate the phytochemical contents, antioxidant and pharmacological activities of its renewable parts using *in vitro*-based assays.

1.13 GENERAL OVERVIEW OF THE THESIS

This thesis is presented in seven Chapters, namely a combined introduction and literature review (Chapter 1), six research chapters (Chapters 2-6) and general conclusions (Chapter 7).

CHAPTER 1, 'Introduction and literature review', provides an introduction on the importance of *S. birrea* and the justification for conservation. The literature review section explores the potential of plant tissue culture technology in the propagation of valuable plants, and also highlights the challenges encountered in the *in vitro* propagation of woody plants. A comprehensive review of the micropropagation of Anacardiaceae species of economic importance is provided. Finally, the value of plant secondary metabolites in medicinal plants and their pharmacological activities is discussed.

In **CHAPTER 2**, 'Conventional seed germination of *Sclerocarya birrea'*, the effects of environmental conditions such as temperature and light on conventional seed germination is explored. An understanding of the conventional seed germination biology of *S. birrea* was necessary since the subsequent *in vitro* propagation experiments were based on seedling explants.

CHAPTER 3, '*In vitro* morphogenesis of nodular meristemoids, describes morphogenesis of nodular meristemoids initiated from leaf explants, and discusses the potential of liquid cultures in mass propagation of *S. birrea*. The advantages of nodule culture are highlighted in comparison to somatic embryogenesis.

CHAPTER 4, ‘Caulogenesis and rhizogenesis in *S. birrea’*, is an investigation on shoot and root formation in *S. birrea* shoots under the influence of different cytokinins and auxins. The *in vitro* organogenesis of hypocotyls and epicotyls is also explored.

CHAPTER 5, ‘Phytochemical and antioxidant evaluation’, evaluates the phytoconstituents of *S. birrea*, and discusses their potential in developing natural antioxidants.

CHAPTER 6, ‘Pharmacological evaluation’, explores the therapeutic value of *S. birrea* extracts using *in vitro* assays for its renewable plant parts. The observed pharmacological activities provide practical possibilities for plant conservation through plant part substitution.

CHAPTER 7, ‘General conclusions’, provides a concise analysis of the implications of the findings of the study.

CHAPTER 2
CONVENTIONAL GERMINATION OF *SCLEROCARYA BIRREA*

2.0 INTRODUCTION

Sclerocarya birrea is one of the most highly-valued indigenous trees in southern Africa **(VON TEICHMAN and ROBBERTSE 1986)**. Besides a number of medicinal uses **(ELOFF 2001)**, the importance of *S. birrea* is underpinned by its diverse characteristics such as high levels of vitamin C and protein, quality stable oil and the novel flavour of its fruit. The vitamin C content of *S. birrea* fruit juice is approximately four to five times more than the levels found in the average orange juice **(JAENICKE and THIONG'O 2000; MOJEREMANE and TSHWENYANE 2004)**. Biochemical analyses indicate that the kernels have higher protein and oil content than most of the popular nuts, including walnuts, hazelnuts, chestnuts, and almonds **(WYNBERG et al. 2003)**.

Mankind has benefited from *S. birrea* as a source of nutrition for more than 10,000 years **(NWONWU 2006)**. Apart from contributing to rural diets, the fruit is used to brew an alcoholic beverage with an annual gross value of about US$ 80-100 per household **(EMANUEL et al. 2005; SHACKLETON et al. 2008)**. The oil is in high demand in the pharmaceutical and cosmetic industries **(NWONWU 2006; KLEIMAN et al. 2008)**, whilst the nuts are used in the food industry for making a range of products including chocolates. With the realisation of its market value, there is notable growth in the trade of *S. birrea* products stimulated by local and industrial demand **(EMANUEL et al. 2005)**.

As industrial demand for its products is increasing, there is a growing concern about the sustainable supply **(NWONWU 2006)** and conservation of wild populations. Thus in the quest for the domestication and improvement of *S. birrea*, understanding of seed germination of this plant is essential. Several members of the Anacardiaceae, including *Sclerocarya* are characterised by a drupe fruit with a stony endocarp. The endocarp of *Sclerocarya* and other related genera has a specialised structure, the operculum, through which the germinating embryo emerges **(VON TEICHMAN and ROBBERTSE 1986)**. According to **HILLS (1933)**, the Anacardiaceae species exhibit

some remarkable seed protection mechanisms by means of a hard lignified endocarp, and intriguingly the most ingenious devices to allow emergence of the germinating embryos. This ingenious opening device (operculum) represents one of the most sophisticated opening mechanisms in the germination of seeds **(VON TEICHMAN and ROBBERTSE 1986)**. Typical of the Anacardiaceae, the germinating unit (nut) in *S. birrea* is the true seed plus endocarp **(LI et al. 1999; GAMÉNÉ et al. 2004)**. The word 'seed' will be used to refer to the true seed.

Although considerable research has been undertaken on this species, there is still a dearth of knowledge on some aspects of its seed biology. **GAMÉNÉ et al. (2004)** inconclusively suggested that seed after-ripening; a decrease in mechanical resistance of the operculum after storage, or a combination of both factors can improve germination of *S. birrea* seeds. Another research gap relates to the effect of light on the germination process **(VON TEICHMAN et al. 1986)**. Generally, under natural conditions, temperature, light, water, oxygen, and mechanical pressures are some of the important factors that can influence seed germination of species like *S. birrea*. The purpose of this study was to identify the possible environmental and mechanical cues influencing the germination of *S. birrea* seeds.

2.1 MATERIALS AND METHODS

2.1.1 SEED COLLECTION

Ripe fruits of *S. birrea* were collected in February 2007 from the Mpumalanga Province of South Africa. Fruits were de-pulped and cleaned as described in the seed leaflet of the **DANIDA FOREST SEED CENTRE (2003)**. The nuts (endocarps) were separated from the pulp, washed, dried, and stored in brown paper bags at room temperature (22 ± 2 °C) for 4 weeks before being tested for germination ability. Seeds used to determine the initial moisture content were not stored. Subsequent marula nuts were received already depulped and were stored as described earlier.

2.1.2 SEED GERMINATION

Before each germination test, nuts were surface-decontaminated by soaking for 15 min in 0.5% (w/v) solution of mercuric chloride ($HgCl_2$). Subsequently, the nuts were

thoroughly rinsed under tap and then distilled water. Before the germination experiments, nuts were soaked for 24 h in the dark at room temperature (22 ± 2 °C), in distilled water (covering 75% of the nut) for the hard endocarps to imbibe water. Seed germination was carried out on cotton wool moistened with distilled water and placed in plastic containers (10.5 X 10.5 X 13.5 cm) in growth chambers equipped with cool white fluorescent lamps (Osram L 58W/640, Germany) emitting a photosynthetic photon flux density (PPFD) of approximately 100 µmol m^{-2} s^{-1} over a wavelength band of 400-700 nm. Light intensity was measured with a quantum radiation sensor (Model Skp 215, Skye Instruments Ltd, UK). Seeds were considered to have germinated when the radicle had emerged at least 2 mm **(BEWLEY 1997)**. Each treatment consisted of 25 seeds and was replicated 4 times. All the experiments were repeated twice. Germination was recorded daily. The nuts which were subjected to continuous dark conditions were examined under a 'green safe light' (wavelength of 510 nm and PPFD of 0.2 µmol m^{-2} s^{-1}) in the dark **(KULKARNI et al. 2006)**. Nuts which were not treated served as a control unless mentioned otherwise. Unless stated otherwise, the duration of germination experiments was 14 days.

2.1.3 MOISTURE CONTENT DETERMINATION

Seeds (embryonic axis and cotyledons) (Fig. 2.1) were excised from the stony endocarp of marula nuts and moisture content was determined gravimetrically by weighing before and after oven-drying at 110 °C for 48-96 h, until a constant weight was obtained. Moisture content was calculated on the basis of fresh weight, (% moisture content) = (Fresh weight - Dry weight)/Fresh weight X 100. The results represent the means of the moisture content values of 20 embryos (± SE) obtained from two separate experiments.

2.1.4 WATER UPTAKE

Water uptake was determined using both intact and opercula-removed (Fig. 2.1) nuts that had been stored at ambient room temperature in the dark for 12 months (see 2.1.6). Intact and opercula-removed nuts (25 seeds per replicate), were placed on cotton wool moistened with distilled water in plastic containers (10.5 X 10.5 X 13.5 cm) and incubated under cool fluorescent white light (16 h light: 8 h dark) with a

PPFD of 60 µmol m^{-2} s^{-1} at room temperature (22 ± 2 °C). Nuts were blotted dry with paper towel, weighed, and replaced in containers at 4 h intervals for 96 h. Percentage water uptake was calculated on the basis of actual increase in mass over the initial mass of the nut **(HIDAYATI et al. 2001)**:

$$\%W_s = [(W_i - W_d)/W_d] \text{ X } 100 \qquad \text{Equation 2.1}$$

where W_s = increase in mass of nuts; W_i = mass of nuts after a given interval of imbibition; and W_d = initial mass of nuts.

2.1.5 EFFECT OF TEMPERATURE

Intact nuts were soaked in distilled water for 24 h before exposing them to different temperatures. Seed germination was determined for both intact and opercula-removed nuts (Fig. 2.1B and C) under alternating light (16-h photoperiod of 100 µmol m^{-2} s^{-1}) and continuous dark conditions (containers were wrapped with aluminium foil). The nuts were incubated at constant temperatures of 10, 15, 20, 25, 30, 35, and 40 °C and alternating temperature of 30/15 °C (14 h/10 h) in plant growth chambers (Controlled Environments Ltd, Manitoba, Canada). The optimum temperature for germination was determined on the basis of constant temperatures using the formula **(KULKARNI et al. 2006)**:

$$T_o = \sum tp / \sum p \qquad \text{Equation 2.2}$$

where T_o is the optimum temperature for germination, and p is the percentage germination at temperature t.

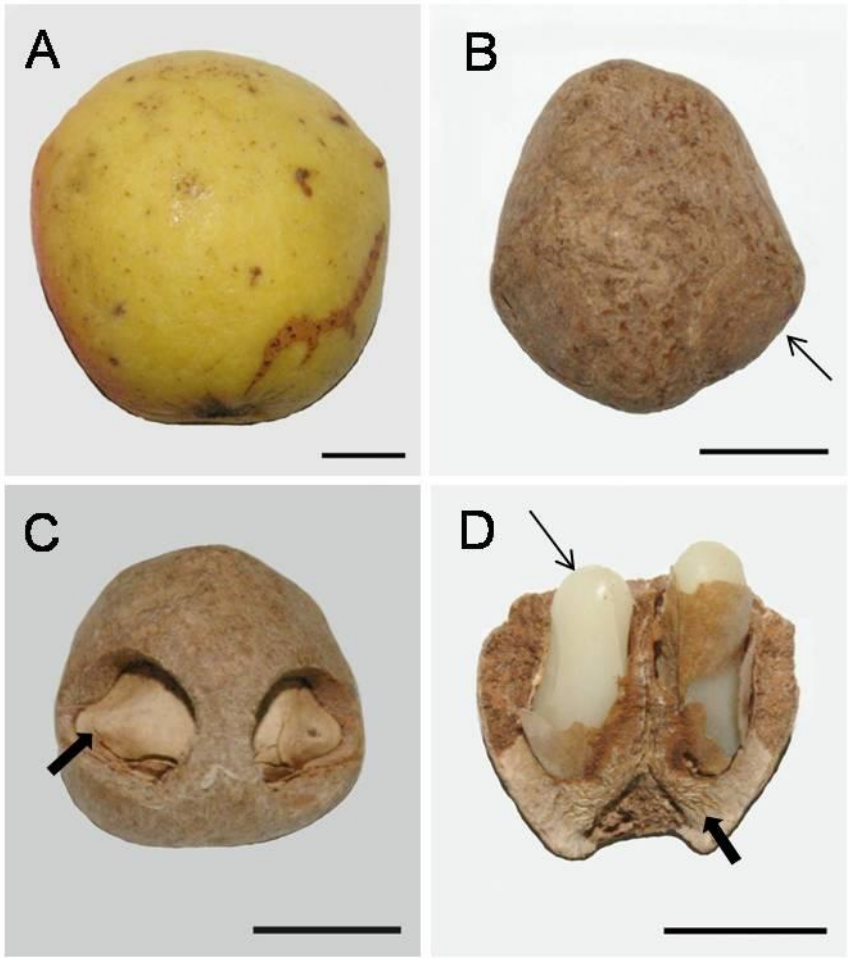


FIGURE 2.1 Structure of a marula fruit and nut. **(A)** Ripe marula fruit. **(B)** An intact marula nut showing the hard lignified endocarp after removal of the fruit pulp. The weight of an intact marula endocarp after 12 months of storage at room temperature was 5.06 ± 0.45 g (*n* = 100). The arrow indicates the operculum, a potential physical barrier to germination, through which the radicle emerges. The weight of an operculum was 0.195 ± 0.025 g (*n* = 100). **(C)** An opercula-removed marula nut with two seeds. The solid arrow indicates the position of the embryo. **(D)** Vertical section of a marula nut showing the walls of the stony endocarp (solid arrow) and the true seeds (fine arrow). Scale bar = 10 mm.

2.1.6 EFFECT OF SEED AFTER-RIPENING ON GERMINATION

Intact nuts with a moisture content of 11.1 ± 1.6% (fresh weight basis) were stored in closed brown paper bags for 6, 9, and 12 months in the dark at room temperature (22 ± 2 °C) following which germination was evaluated. At each interval 6, 9 and 12 months nuts were removed from storage and tested for their germination response to

temperature and light. For cold and warm stratification experiments only nuts stored for 12 months were tested for germination.

2.1.7 EFFECT OF LIGHT SPECTRA ON GERMINATION

To examine the effect of different light spectra, nuts were placed in boxes fitted with red (1.5 µmol m^{-2} s^{-1}), far-red (1.4 µmol m^{-2} s^{-1}), blue (0.2 µmol m^{-2} s^{-1}), and green (0.2 µmol m^{-2} s^{-1}) light filters and incubated under continuous light (100 µmol m^{-2} s^{-1}) using cool white fluorescent lamps (Osram L 58W/640, Germany) at 25 ± 2 °C **(KULKARNI et al. 2006)**. Incubation in the dark served as the control. Percentage germination was recorded after 7 days.

2.1.8 PRE-GERMINATION TREATMENTS

Intact nuts were used for all the pre-germination treatments. For all pre-germination experiments, controls consisted of untreated nuts. The nuts were incubated on cotton wool moistened with distilled water. For the acid scarification treatment, nuts were soaked in 96% (v/v) sulphuric acid (H_2SO_4) for 2, 4, 6, 8, and 10 h. The nuts were subsequently rinsed thoroughly in water for 30 min. In another treatment, the nuts were subjected to boiling water for 5, 10, 15, 20, 25, and 30 min, removed and left to cool for 30 min. For the dry heat treatment nuts were placed in the oven and exposed to 110 °C for 2, 4, 6, 8, and 10 h. For soaking treatments nuts were placed in distilled water for 12, 24, 48, 72, 96, and 168 h at ambient room temperature in the dark. After each treatment, the nuts were soaked in distilled water for 24 h (excluding soaking treatments) and incubated in a plant growth chamber (Controlled Environments Ltd, Manitoba) under continuous dark conditions at 25 ± 2 °C. Kinetin, gibberellic acid (GA_3), and potassium nitrate (KNO_3) were tested at 0.1, 0.01, and 0.001 µM concentrations under both continuous light and continuous dark conditions. Nuts were soaked in these solutions for 24 h and incubated on cotton wool moistened with distilled water at 25 ± 2 °C.

2.1.9 SEED STRATIFICATION

After 12 months storage at 22 ± 2 °C intact nuts were placed between two layers of paper towel, moistened with distilled water (using a 500ml plastic spray bottle) and kept inside plastic bags. These bags were then stored in the dark at 5 °C (cold

stratification) and 40 °C (warm stratification) for 7, 14, 21, and 28 days. For each treatment nuts in 4 plastic bags were incubated at the respective temperature. After the respective incubation periods, germination tests were conducted under continuous dark conditions at 25 ± 2 °C. The nuts used for the stratification experiments had been stored for 12 months.

2.1.10 STATISTICAL ANALYSIS

Seed germination data were expressed as mean values ± standard error (SE). The germination percentage data were arcsine transformed before statistical analysis in order to ensure homogeneity of variance. One-way analysis of variance (ANOVA) was conducted and Tukey's test was used to separate differences among treatment means. Data were analysed using SPSS for Windows (version 15, SPSS®, Chicago).

2.2 RESULTS

2.2.1 SEED MOISTURE CONTENT

Sclerocarya birrea nuts stored for 12 months at room temperature had a mean fresh weight of 5.06 ± 0.45 mg (*n* = 100). The moisture percentage of excised true seeds from fresh nuts was 11.1 ± 1.6% which was significantly higher than that of 12-month-old nuts (4.9 ± 0.57%). Assessment of seed water content was done after 48 h at 110 °C.

2.2.2 IMBIBITION

Water uptake by both 12-month-old intact (26.9 ± 1.03%) and opercula-removed (32.2 ± 1.16%) nuts followed a similar imbibition curve, though water uptake for intact nuts was consistently lower (Fig. 2.2). Initially, in both intact and opercula-removed *S. birrea* nuts, the rate of water uptake up to 8 h was rapid and slowed down thereafter.

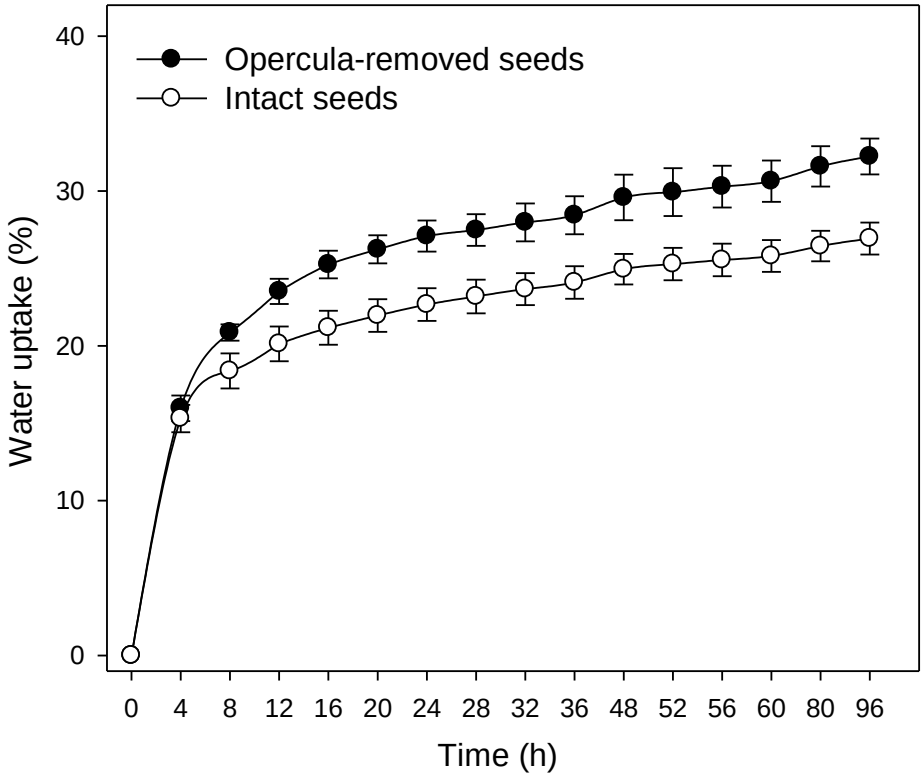


FIGURE 2.2 Water uptake for *S. birrea* nuts during 96 h of incubation under alternating light (16-h photoperiod) at room temperature (*n* = 25).

2.2.3 EFFECT OF TEMPERATURE AND LIGHT ON GERMINATION

At all the temperatures tested, alternating light (16-h photoperiod) was inhibitory for seed germination of 9-month-old opercula-removed nuts. Opercula-removed nuts exhibited higher percentage germination than intact nuts between 20 and 35 °C under constant dark conditions (Table 2.1). Low temperatures (10 and 15 °C) inhibited seed germination of both intact and opercula-removed fruits. At high constant temperature regimes (25-40 °C), 9-month-old intact nuts did not germinate. On the other hand, 12-month-old intact nuts exhibited moderate germination ranging from 20.8 ± 5.3% (25 °C) to 33.2 ± 5.9% (30 °C). A similar trend was noted for alternating temperature (30/15 °C) (Table 2.1). The calculated optimum temperature (T_o) for germination was 29 °C.

After 6 months of storage, percentage germination of opercula-removed nuts was significantly higher under continuous dark in comparison to both continuous and

alternating light conditions where no germination was recorded (Fig. 2.3A). However, the inhibitory effect of white light on seed germination was significantly reduced after 12 months of storage, exhibiting 65.2 ± 0.9% and 67.2 ± 1.3% germination under continuous and alternating light conditions, respectively (Fig. 2.3B). Intact nuts stored for up to 12 months germinated equally in continuous dark (31.3 ± 4.4%), continuous light (32.7 ± 2.6%), and alternating light (30.5 ± 3.8%) conditions (Fig. 2.3C). The photoinhibitory effect on germination was lost with prolonged storage of the marula nuts under ambient conditions in the dark.

Red and blue light spectra had a stimulatory effect on the germination of 6-month-old opercula-removed nuts (Table 2.3). However, the sensitivity of opercula-removed nuts to different light spectra was reduced after prolonged seed storage of 12 months (Table 2.3).

TABLE 2.1 Effect of different temperatures on germination of seeds stored for nine or 12 months under constant dark conditions

Temperature (ºC)	Germination (%)			
	9-month-old nuts		12-month-old nuts	
	Opercula-removed	Intact	Opercula-removed	Intact
10	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
15	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
20	52.6 ± 6.8 b	0 ± 0 a	54.5 ± 4.3 b	6.2 ± 4.0 ab
25	91.3 ± 1.5 c	0 ± 0 a	94.8 ± 1.2 c	20.8 ± 5.3 bc
30	87.0 ± 2.9 c	0 ± 0 a	90.6 ± 1.8 c	33.2 ± 5.9 c
35	87.0 ± 6.4 c	0 ± 0 a	86.2 ± 5.8 c	20.8 ± 7.9 bc
40	25.0 ± 5.9 ab	0 ± 0 a	26.2 ± 6.1 ab	31.2 ± 8.5 bc
30/15	86.4 ± 5.1 c	0 ± 0 a	92.4 ± 4.2 c	18.0 ± 3.4 bc

Values (± SE) with different letters in a column are significantly different at 5% level of significance according to Tukey's test ($p \leq 0.05$).

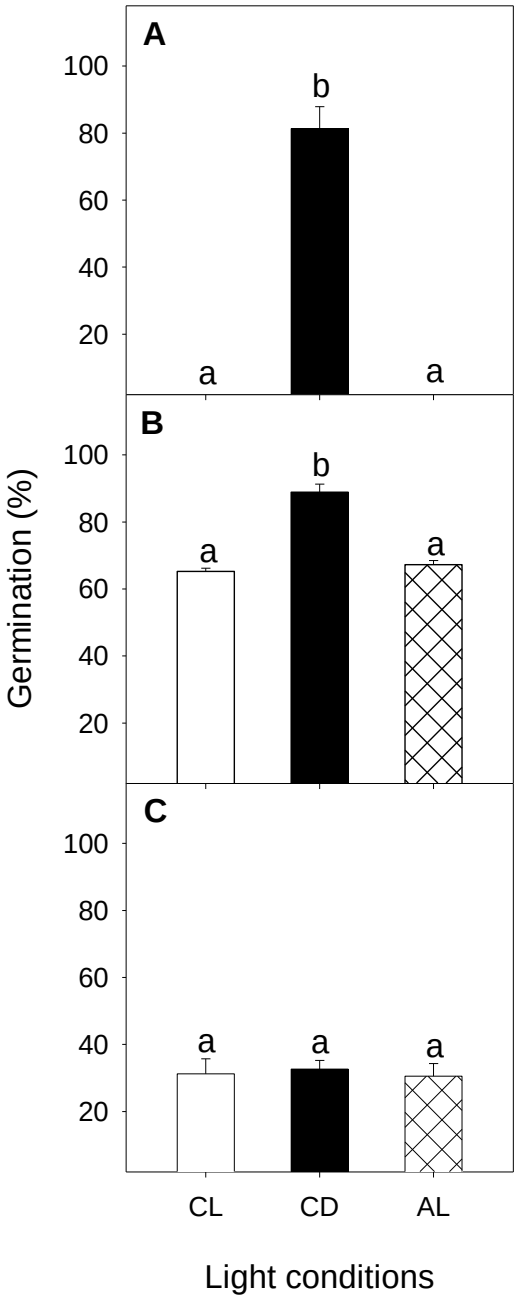


FIGURE 2.3 Effect of light conditions and seed storage on germination (%) of *S. birrea* nuts incubated at 25 ± 2 °C. CL = continuous light, CD = continuous dark, and AL = alternating light (16-h photoperiod). **(A)** Germination of opercula-removed nuts after a 6 months storage period. **(B)** Germination of 12-month-old opercula-removed nuts. **(C)** Germination of intact nuts after a 12 month storage period. The opercula were removed just before the germination tests. Bars (± SE) with similar letters are not significantly different at 5% level of significance (Tukey's test, $p \leq 0.05$).

TABLE 2.2 Effect of different light spectra on seed germination

Light source	Germination (%)	
	6-month-old nuts	12-month-old nuts
Dark (control)	81.3 ± 6.5 d	83.3 ± 0.0 b
White light	0 ± 0.0 a	79.1 ± 4.1 b
Red	53.2 ± 7.5 cd	80.5 ± 8.6 b
Far-red	32.8 ± 12.1 cb	61.1 ± 6.3 a
Green	24.7 ± 5.1 b	61.1 ± 2.4 a
Blue	46.6 ± 17.8 c	77.7 ± 2.4 b

Values (± SE) with different letters in a column are significantly different at 5% level of significance according to Tukey's test ($p \leq 0.05$).

2.2.4 EFFECT OF TEMPERATURE AND LIGHT SHIFTS

Opercula-removed nuts (9-month-old) that did not germinate under a 16-h photoperiod at a temperature of 25 °C were moved to continuous dark conditions at 25 °C resulting in the reversal of the photoinhibition effect (Fig. 2.4). Similarly, seeds that did not germinate at 10, 15 and 20 °C showed high germination percentages when shifted to 25 °C in the dark (Fig. 2.4).

2.2.5 EFFECT OF AFTER-RIPENING ON GERMINATION

Intact nuts of *S. birrea* germinated to 33.2 ± 5.9% at 30 °C under constant dark conditions after a 12 months after-ripening period (Table 2.1). White light inhibited germination of 6-month-old opercula-removed nuts under both continuous and alternating light conditions. However, dark conditions promoted germination of *S. birrea* irrespective of the after-ripening period of the nuts (Fig. 2.3A and 2.3B). After 12 months of storage, the photoinhibition effect was partially lost with significant improvement in final germination (Fig. 2.3B).

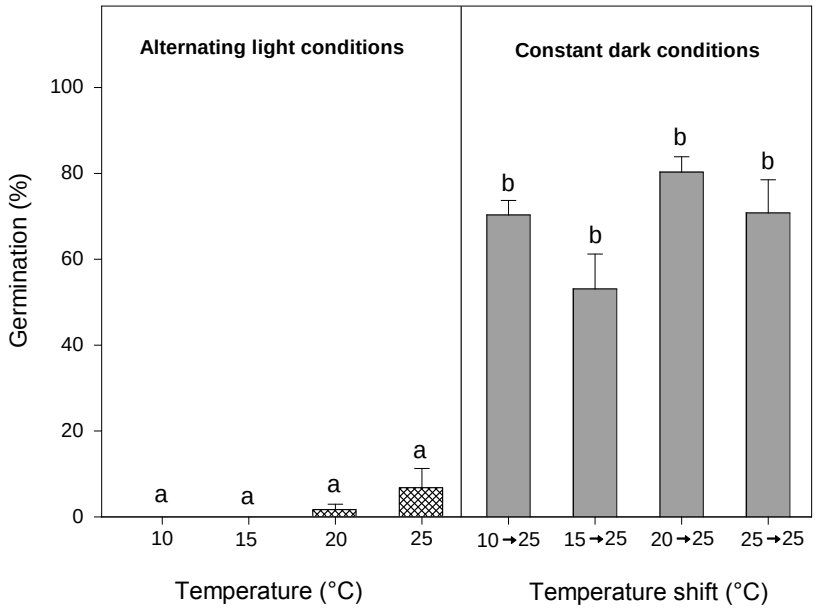


FIGURE 2.4 Temperature and light shift effects on percentage germination of *S. birrea* nuts after a storage period of 9 months. The nuts which had been after-ripened for 9 months were shifted from alternating light to constant dark conditions after 14 days. As shown, the inhibitory light effect was reversible. Bars (± SE) with similar letters are not significantly different at 5% level of significance (Tukey's test, $p \leq 0.05$).

2.2.6 EFFECT OF PRE-GERMINATION TREATMENTS

All pre-germination experiments were conducted on 12-month-old nuts. Scarification with sulphuric acid (H_2SO_4) did not improve germination of *S. birrea* seed relative to the control (data not shown). Boiling water and dry heat also did not improve seed germination of *S. birrea* in comparison to the controls. The germination for dry heat and boiling water treatments was 0% indicating that the seeds probably were killed. Furthermore, neither prolonged soaking ($p > 0.420$) nor application of plant growth regulators ($p > 0.665$) and KNO_3 ($p > 0.882$) were effective in enhancing germination of *S. birrea* seeds.

2.2.7 COLD STRATIFICATION

Sclerocarya birrea nuts subjected to cold stratification for a period of 14 days at 5 °C showed significantly greater germination (65%) compared to non-stratified (32%) and nuts that were cold stratified for 7, 21 and 28 days (< 32%) (Fig. 2.5). Warm stratification (results not shown) did not improve seed germination (*p > 0.348*).

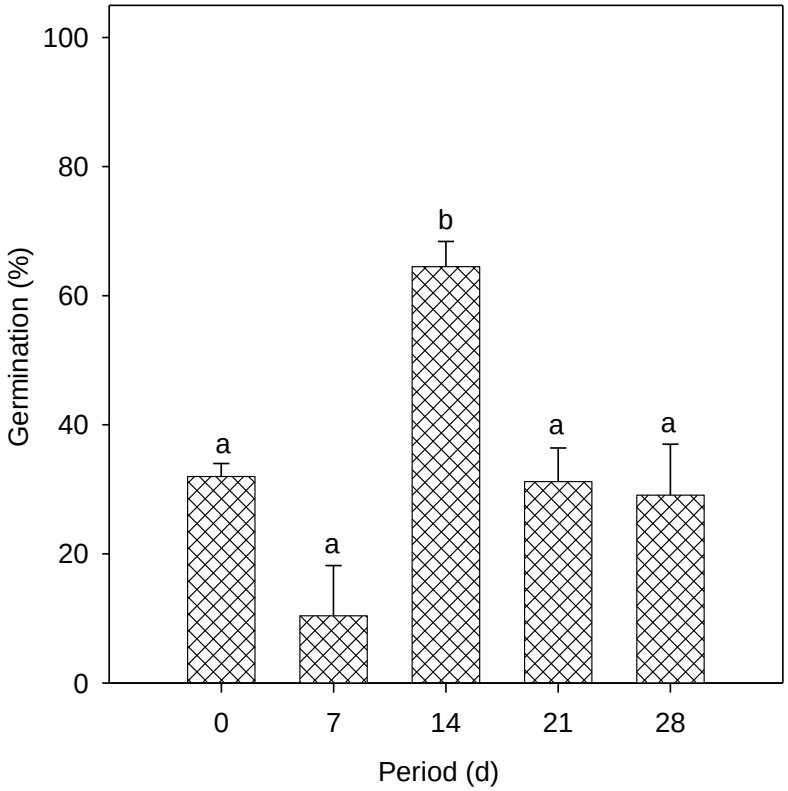


FIGURE 2.5 Effect of cold stratification (5 °C) on germination of intact *S. birrea* nuts under continuous dark conditions at 25 ± 2 °C. The nuts were cold-stratified after a 12 months storage period. Bars (± SE) with similar letters are not significantly different at 5% level of significance (Tukey's test, $p < 0.05$).

2.3 DISCUSSION

Storage of marula nuts under dark conditions at room temperature for 12 months resulted in moisture loss from the seeds. The difference in seed moisture content between fresh and 12-month-old nuts could explain the higher germination percentage following the prolonged storage period. On the basis of its tolerance to

low moisture content of 4.9 ± 0.57%, *S. birrea* seeds can be classified as orthodox. This confirms the findings of **PRITCHARD et al. (2004)**. According to **PRITCHARD et al. (2004)** and **TWEDDLE et al. (2003)**, desiccation-tolerant or orthodox seeds can tolerate low moisture content below 7% and subsequently rehydrate without significant variation in viability. Generally, seeds of most trees and shrubs adapted to arid and highly seasonal environments are overwhelmingly desiccation tolerant **(TWEDDLE et al. 2003)**.

Sclerocarya birrea seeds are relatively nutrient-rich **(SHACKLETON et al. 2003; WYNBERG et al. 2003)**. Accordingly, the large *S. birrea* nut exhibits typical characteristics of species found in dry tropical regions, where seeds have relatively large quantities of nutrients, to support rapid growth of seedlings, thereby increasing their chances of survival **(TWEDDLE et al. 2003)**. Both intact and opercula-removed *S. birrea* nuts readily imbibe water (Fig. 2.2). Intact *Lannea microcarpa* (also Anacardiaceae) seeds were found to imbibe water readily although the rate of water uptake was faster for seeds with scarified endocarps **(NEYA et al. 2008)**. In contrast, the seeds of other species of Anacardiaceae such as *Rhus aromatica* and *R. glabra* were not permeable to water, even when their outer two layers (brachysclereids and osteosclereids) were removed **(LI et al. 1999)**.

As *S. birrea* is adapted to the drier tropical regions, high temperatures in the range of 25 to 35 °C favour seed germination. In this study, the calculated optimum temperature (T_o) for the germination of *S. birrea* seeds was 29 °C under constant dark conditions. Fig. 2.4 depicts the effect of both light and temperature shifts for *S. birrea* nuts after 9 months storage. When opercula-removed nuts did not germinate at 10 and 15 °C or showed little germination at 20 and 25 °C under 16-h photoperiod, they were shifted to continuous dark at 25 °C, which significantly increased percentage germination. Although sustained dark exposure promoted germination best, continuous exposure of seeds to specific light spectra compared with white light had a significant influence on percentage germination of 6-month-old nuts of *S. birrea* (Table 2.2). In particular, red and blue light increased seed germination as compared to white light for the 6-month-old nuts.

These results may indicate the influence of the phytochrome family of photoreceptors on seed germination. The inhibitory effect of white light may be due to a high rate of interconversion between the P_{fr} and P_r forms of phytochrome caused by high PPFD of light of any wavelength **(ELLIS et al. 1989)**. This high irradiance reaction (HIR) overrides the reversible phytochrome reactions and may be inhibitory to seed germination **(BASKIN and BASKIN 1998)**. The stimulatory influence on seed germination of both red and blue light observed for 6-month-old nuts was lost, as was the inhibitory effect of white light, with prolonged storage of the marula nuts (12 months) at room temperature (Table 2.2).

Another factor influencing seed germination is after-ripening in storage. **VON TEICHMAN et al. (1986)** and **GAMÉNÉ et al. (2004)** reported that final germination of *S. birrea* seeds increased following one year and six months of storage under ambient temperature and relative humidity conditions. This shows that after-ripening of seeds during storage at ambient temperatures is critical for germination of *S*. *birrea*. Using opercula-removed nuts, **PRITCHARD et al. (2004)** showed that physiological rather than physical dormancy had a predominant influence on seed germination of *S*. *birrea*. For *L. microcarpa*, the increase in germination with drying was attributed to seed after-ripening and/or a loss of physiological dormancy **(NEYA et al. 2008)**. It has further been suggested that desiccation tolerance is greatest for seeds exhibiting physical and combinational (physical and physiological) dormancy **(TWEDDLE et al. 2003)**.

Intact nuts of *S. birrea* stored up to 6 months failed to germinate under all the photoperiod conditions (continuous light, continuous dark, and alternating light) examined. However, after 12 months of storage there was a partial loss of dormancy (Fig. 2.3C). Similarly for *Prosopis juliflora*, storage of seeds achieved significantly greater and faster germination **(EL-KEBLAWY and AL-RAWAI 2006)**. The germination response of *S. birrea* seeds to light and storage suggests both physiological and endocarp-imposed dormancy.

VON TEICHMAN et al. (1986) showed that acid scarification was not effective in enhancing seed germination of *S*. *birrea*. However, **GAMÉNÉ et al. (2004)**

reported an increase in germination after treating the marula nuts with hydrochloric acid (HCl). **LI et al. (1999)** have reported that concentrated H_2SO_4 released seed dormancy of *R. aromatica*, and boiling water that of *R. glabra*, both members the Anacardiaceae. For *S. birrea* scarification with H_2SO_4, boiling water, dry heat and prolonged soaking of nuts did not improve seed germination.

Seeds of some species with stony endocarps germinate better when they are subjected to different periods of cold stratification, for example, *Cornus* sp. (90-120 days), *Coryllus* sp. (60-180 days), *Menispermum* sp. (14-28 days), *Morus* sp. (30-90 days), *Nyssa* sp. (30-120 days), and *Oemleria* sp. (120 days) **(YOUNG and YOUNG, 1992)**. For *S. birrea* seeds, a cold stratification treatment of 14 days significantly increased germination (Fig. 2.5). The response to cold stratification indicates adaptive mechanisms and significance of natural environmental cues such as low winter temperatures, to which *S. birrea* seeds may be exposed before germination.

2.4 CONCLUSIONS

This study has identified the factors that influence the germination of *S. birrea*. Both intact and opercula-removed nuts readily imbibe water suggesting physiological rather than physical dormancy. Light had an inhibitory effect on seeds of opercula-removed nuts, which was subsequently eliminated after prolonged storage at ambient temperature. Seeds of *S. birrea* can be considered as orthodox as they tolerated desiccation. The findings of this study indicate that after-ripening, light, temperature, and cold stratification are critical determinants for the germination of *S. birrea* seeds.

CHAPTER 3
IN VITRO MORPHOGENESIS OF LEAF-DERIVED NODULAR MERISTEMOIDS

3.0 INTRODUCTION

Sclerocarya birrea is an indigenous tree that is highly valued for its medicinal and nutritional properties by local communities. Marula products are also in high demand in the pharmaceutical, cosmetic and food industries. With the increasing demand of marula products, micropropagation can become a viable option in the conservation, propagation and breeding of the plant. The most commonly used micropropagation techniques include direct and indirect shoot organogenesis, and somatic embryogenesis. Somatic embryogenesis is a more desirable biotechnological technique for large-scale clonal propagation of elite genotypes, development of synthetic seed technology, plant transformation **(BANDYOPADHYAY and HAMILL 2000; BOMAL and TREMBLAY 2000)** and conservation of threatened plant species. The most ideal regeneration systems for genetic transformation are direct and repetitive production of somatic embryos or *de novo* shoot organogenesis originating from single cells in the epidermis **(RUGKHLA and JONES 1998)**. Whereas genetic clones created through shoot organogenesis require further manipulation for root initiation, in somatic embryogenesis complete plants with a bipolar axis, vascular system and functional meristems are produced in a single step **(BASSUNER et al. 2007)**. In somatic embryogenesis, somatic cells develop into plants in similar developmental stages to zygotic embryogenesis **(GOMES et al. 2006)**. Recently, several reports have described shoot organogenesis through somatic embryo-like structures from different explants, mainly leaves and hypocotyls. Histological observations of the embryo-like structures have confirmed the initial globular-shaped morphological features **(HAENSCH 2004; TIAN et al. 2008)** during the early developmental stages of their growth, in synchrony with conventional somatic embryogenesis. These embryo-like structures have in the past been mistaken for true somatic embryos **(SALAJ et al. 2005)**. Recently, **HAENSCH (2004)** working on *Pelargonium* x *hortorum* observed that although globular and heart-shaped embryo-like regenerants were formed, histological analysis showed

that they lacked a defined root pole. In contrast to somatic embryogenesis where the globular-stage normally consists of small cells with dense cytoplasm and large nuclei **(HAENSCH 2004)**, globular embryo-like structures had vacuolised parenchymatous cells **(SALAJ et al. 2005)**. In *Elliottia racemosa* the regenerants had a broad base of attachment to the maternal tissues, indicating that somatic embryogenesis did not occur **(WOO and WETZSTEIN 2008)**. Similar structures observed in other plant species have also been referred to as meristemoids, promeristems, meristemoid-like precursors **(HICKS 1994)**, protocorm-like bodies **(YOUNG et al. 2000 and TIAN et al. 2008)**, nodules **(McCOWN et al. 1988; ZIV et al. 1994; TENG 1997; BATISTA et al. 2000; XIE and HONG 2001; FERREIRA et al. 2009)** and nodular meristemoids **(McCOWN et al. 1988)**. **McCOWN et al. (1988)** defined nodules as independent, spherical, dense cell clusters which are cohesively bound together and exhibit consistent internal cell/tissue differentiation, and loosely resemble protocorms. On the other hand, a meristemoid can be considered as a cluster of cells acting together as a meristematic centre **(McCOWN et al. 1988)**. For our discussion we have adopted the term nodular meristemoid. A histological approach is important in distinguishing true somatic embryos and nodular meristemoids. Many published reports on somatic embryogenesis have drawn conclusions on the basis of morphological appearance only, and should be re-evaluated critically **(BASSUNER et al. 2007)**. Histological approaches can provide critical information to allow the application of the most appropriate *in vitro* plant regeneration strategies **(WOO and WETZSTEIN 2008)**. This chapter describes the induction and development of nodular meristemoids from leaf explants of *S. birrea* partly using a histological approach to determine the anatomical characteristics.

3.1 MATERIALS AND METHODS

3.1.1 PLANT MATERIALS

Leaf explants were obtained from *S. birrea* seedlings grown in plant growth chambers (Controlled Environments Ltd, Manitoba, Canada) at 25 ± 2 °C under a 16-h photoperiod at a photosynthetic photon flux density of 100 µmol m^{-2} s^{-1} provided by cool white fluorescent light. The explants were rinsed thoroughly in running tap water, before being surface-decontaminated in 70% alcohol for 1 min

followed by 2% sodium hypochlorite for 20 min with a few drops of Tween 20 (polyoxyethylene sorbitan monolaurate, Saarchem, Krugersdorp, South Africa). The explants were then thoroughly rinsed in sterile distilled water 3 times. Leaf explants were cultured with the abaxial surface on the growth medium.

3.1.2 INDUCTION OF NODULAR MERISTEMOIDS

Woody plant medium (WPM; **LLOYD and McCOWN 1981)** and Murashige and Skoog medium (MS; **MURASHIGE and SKOOG 1962)** were supplemented with vitamins, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), polyvinylpyrrolidone (PVP) (3 g l^{-1}) and plant growth regulators and adjusted to pH 5.8 before the addition of the gelling agent (8 g l^{-1} Agar Bacteriological, Agar No.1, Oxoid Ltd, Bastingstoke, England). The media were then autoclaved at 121 °C, 15 KPa for 20 min. The cytokinin, 6-benzyladenine (BA), was used in different concentrations (0, 1.0, 2.0 and 4.0 µM) in combination with three auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) at final concentrations in the medium of 0, 1.0, 2.0 and 4.0 µM. Plant growth regulators were added to the growth medium before autoclaving. All the plant growth regulators were obtained from Sigma-Aldrich, St. Louis, USA. Cultures were maintained at a temperature of 25 ± 2 °C under a 16-h photoperiod. A constant photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} was provided by cool white fluorescent light (Osram L 58W/640, Germany). Light intensity was measured with a quantum radiation sensor (Model Skp 215, Skye Instruments Ltd, Llandridod Wells, Powys, UK). Each treatment consisted of 18 replicates and the experiment was done twice. After 4 weeks in culture data on the percentages of explants that formed nodules, callus and differentiated shoot buds, as well as the mean number of shoots per responding explant were recorded.

3.1.3 PLANTLET REGENERATION

For plantlet development nodular meristemoids were maintained on solid medium or transferred to liquid plantlet development medium consisting of MS medium supplemented with vitamins, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), polyvinylpyrrolidone (3 g l^{-1}), and combinations of BA and NAA or IBA that gave the best results in the culture initiation experiments. The nodules were incubated

at 25 ± 2 °C under a 16-h photoperiod at a photosynthetic photon flux density of 40 µmol m^{-2} s^{-1}. Nodular meristemoids in liquid medium were placed on a shaker at a speed of 120 rpm. Sixteen replicates were done for each treatment in liquid shake culture, and the experiment was repeated twice. The liquid shake culture treatments consisted of eight flasks per treatment with two clusters of nodular meristemoids in each. After 4 weeks plantlet growth was recorded. Data on the number of differentiated shoots and mean shoot length were recorded after a 4-week culture period.

3.1.4 HISTOLOGICAL EXAMINATION

Nodular meristemoids were fixed overnight at 4 °C in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. After rinsing with buffer (twice, 30 min each), the embryos were post-fixed in 2% osmium tetroxide in 0.05 M sodium cacodylate buffer for 10 min **(JAYASANKAR et al. 2003)**. After rinsing twice (30 min each time) in the same buffer solution, the samples were serially dehydrated (10 min each time) in ethanol (30–90%) and three times in 100%. The specimens were infiltrated with propylene oxide (twice for 30 min each), and embedded with a serial increment of Epon/Araldite resin: propylene oxide [25:75, 50: 50, 75:25 and 100: 0 (v/v)]. The samples were polymerised in 100% Epon/Araldite resin and polymerised at 70 °C for 48 h. Blocks of resin, approximately 1–4 mm^3, containing an embryo or cluster of embryos were excised and attached to the solidified resin blanks with superglue **(JAYASANKAR et al. 2003)**. Sections were then cut on an LKB ultrotome III® (Stockholm, Sweden) using glass knives. Sections (5 µm thick) for light microscopy were stained with Ladd Multiple stain (Toluidine blue and basic fuchsin in 30% ethyl alcohol) for 2 min. Excess stain was washed away with distilled water and sections were secured to glass slides with gentle heat at 70 °C. Sections were immediately observed under a fluorescence microscope (Olympus AX70, Olympus, Japan) equipped with a digital capture system with a minimum 300 dpi resolution.

3.1.5 MACROSCOPIC EVALUATION

Macroscopic images were recorded using a stereomicroscope (Leica MZ16, Switzerland) fitted with a digital image capture system with a minimum resolution

of 300 dpi **(GOMES et al., 2006)**. Samples for scanning electron microscopy were fixed in cold 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2 and incubated overnight at 4 °C. The nodules were washed twice at 30 min each time in the same buffer, and dehydrated through a graded ethanol series as described earlier. The samples were critical point dried with carbon dioxide using a HCP-2 critical point dryer (Hitachi, Japan), mounted on aluminium stubs and sputter-coated with a 20 nm layer of gold-palladium. The specimens were immediately examined with a Hitachi S–570 scanning electron microscope (Hitachi, Japan) operating at 7–8 kV.

3.1.6 EXPERIMENTATION AND DATA ANALYSIS

Data were recorded after 4 weeks on the frequency of nodular meristemoid induction, shoot regeneration and callus formation. In addition, some of the nodular meristemoids were maintained on the same medium or re-cultured in liquid medium and evaluated for shoot regeneration after 4 weeks. The experiments were arranged in a completely randomised design and each experiment was repeated twice. Percentage data were arcsin transformed before being statistically analysed. One-way analysis of variance (ANOVA) was done and Tukey's test was used to separate differences among treatment means ($p \leq 0.05$). Data were analysed using SPSS for Windows (version 15, SPSS®, Chicago).

3.2 RESULTS

3.2.1 INDUCTION OF NODULAR MERISTEMOIDS

After 2 weeks of culture, leaf explants on media supplemented with plant growth regulators showed signs of expansion and the formation of small globular-shaped structures. The globular shaped structures became more evident after 3 weeks of culture and showed resemblance to the globular stage of true somatic embryos. The clusters of nodular meristemoids became more defined after 4 weeks of culture (Fig. 3.1B). Some leaf-like structures were also observed at this stage in some culture tubes (Fig. 3.5A). The embryo-like morphology was confirmed by microscopy (Fig. 3.5A) and scanning electron microscopy (Fig. 3.5B). High frequencies of nodular clusters were regenerated on WPM supplemented with BA

in combination with NAA, IBA or IAA. On MS medium the induction of nodular clusters was relatively high for BA and NAA combinations and low when BA was combined with IBA or IAA (Table 3.1). As indicated in Table 3.1 lower concentrations of BA (1.0 or 2.0 µM) in combination with 1.0 µM NAA resulted in higher nodular meristemoid induction frequency on WPM (83–86%) when compared to the same combinations on MS medium (38–66%). The highest nodular meristemoid induction on MS medium was obtained with supplementation of 4.0 µM BA and 1.0 µM NAA (Table 3.1). On woody plant medium, supplementation with 1.0 or 4.0 µM BA and 1.0 µM IBA resulted in 100% nodule induction. In general, combinations of BA and IBA gave high frequencies of nodular meristemoid induction on WPM as compared to MS medium (Table 3.1). A similar trend was also observed for BA and IAA, although no shoots were subsequently regenerated on WPM. The largest nodular meristemoids were observed on MS medium supplemented with 4.0 µM BA and 1.0 µM NAA (Fig. 3.1B). The highest percentage of explants (66.6%) that produced callus was observed on 1.0 µM BA and 4.0 µM IBA. However, the callus did not differentiate into shoots.

3.2.2 PLANTLET REGENERATION

When cultures were maintained on the same medium for a further 4 weeks, the conversion rate from the nodular meristemoid clusters into fully differentiated shoots was low (Table 3.1). The highest conversion rate (22%) was achieved on MS medium supplemented with 4.0 µM BA and 1.0 µM NAA (Table 3.1). For some of the experimental treatments plantlets developed from nodular meristemoid clusters (Fig. 3.2 and 3.3). The media and plant regulator combinations in which shoot organogenesis was observed on solid medium were used for the liquid culture experiments. There was an improvement on shoot induction when nodular meristemoid clusters were transferred to liquid shake culture (Fig. 3.4). The highest rate of conversion of nodular clusters into plantlets was 62% on MS medium supplemented with 2.0 µM BA and 1.0 µM NAA (Table 3.2). The longest shoots (23.5 ± 6.2 mm) were obtained on MS medium with 4.0 µM BA and 1.0 µM NAA after 4 weeks in liquid medium (Table 3.2).

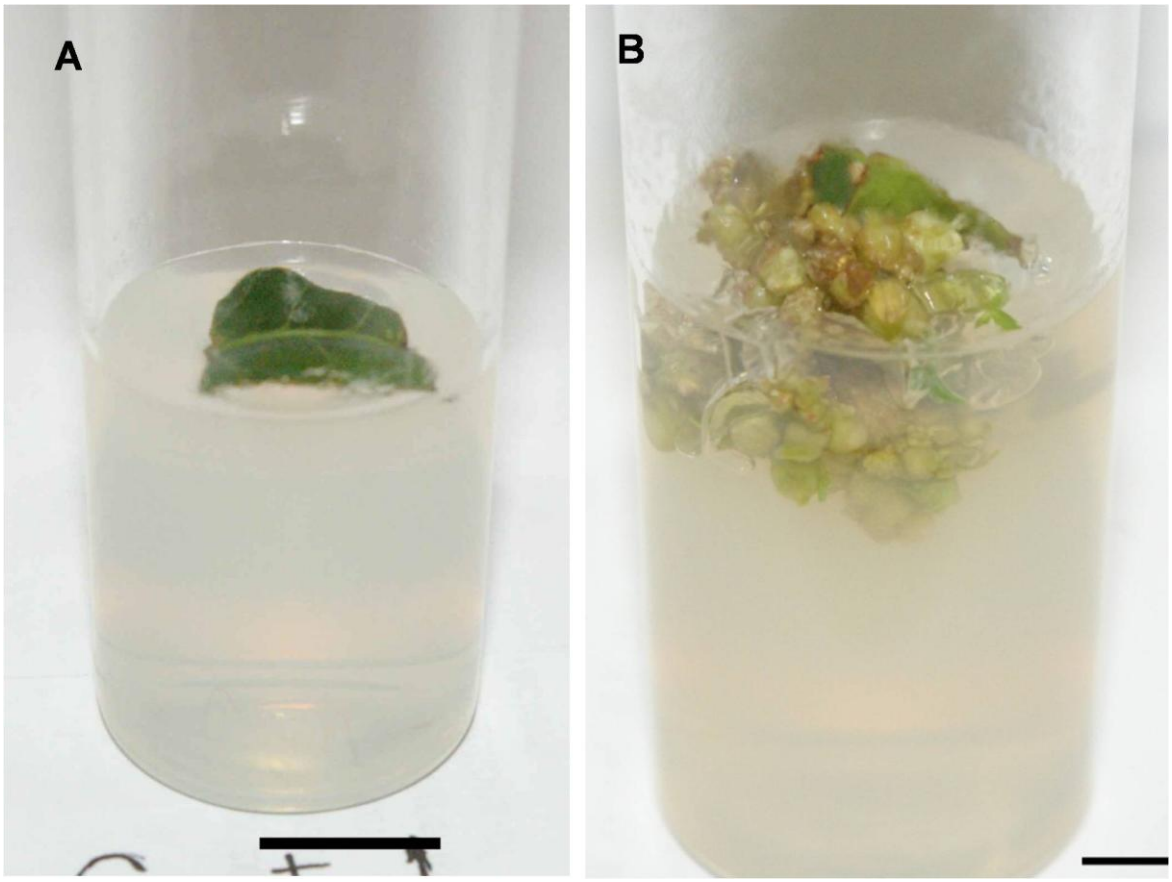


FIGURE 3.1 Plant regeneration from *S. birrea* leaf explants on different concentrations of BA in combination with NAA. **(A)** In the absence of plant growth regulators the control did not respond after 4 weeks in culture. **(B)** Nodular meristemoids induced on MS supplemented with 4.0 µM BA and 1.0 µM NAA after 4 weeks of culture. Scale bar = 10 mm.

TABLE 3.1 Effects of BA and auxins on nodule induction and shoot regeneration of *S. birrea* leaf explants after 4 weeks in culture

Medium	Cytokinin (µM)	Auxin (µM)			Callus induction (%)	Nodule induction (%)	Shoot bud formation (%)	No. of shoots/ explant[1]
MS	BA	NAA	IBA	IAA				
	1.0	1.0	-	-	0 a	38.8 cdef	0 f	0 a
	2.0	1.0	-	-	0 a	66.6 hi	19.4 ef	1.3 ab
	4.0	1.0	-	-	11.1 b	86.1 jk	22.2 f	3.5 c
	1.0	2.0	-	-	13.8 b	0 a	0 a	0 a
	1.0	4.0	-	-	27.7 c	0 a	0 a	0 a
	1.0	-	1.0		52.7 d	30.5 bcd	0 a	0 a
	2.0	-	1.0		47.2 d	33.3 bcde	0 a	1.5 ab
	4.0	-	1.0		52.7 d	44.4 defg	8.3 b	1.0 ab
	1.0	-	2.0		27.7 c	44.4 defg	0 a	0 a
	1.0	-	4.0		66.6 e	33.3 bcde	0 a	0 a
	1.0	-	-	1.0	0 a	0 a	0 a	0 a
	2.0	-	-	1.0	30.5 c	24.9 bc	13.8 cd	0 a
	4.0	-	-	1.0	36.1 c	49.9 efg	13.8 cd	0 a
	1.0	-	-	2.0	33.3 c	16.6 ab	0 a	0 a
	1.0	-	-	4.0	27.7 c	0 a	0 a	0 a
WPM	1.0	1.0	-	-	0 a	86.1 jk	0 a	0 a
	2.0	1.0	-	-	0 a	83.3 jk	0 a	0 a
	4.0	1.0	-	-	47.2 d	49.9 efg	0 a	0 a
	1.0	2.0	-	-	11.1 b	52.7 fg	0 a	0 a
	1.0	4.0	-	-	13.8 b	44.4 defg	0 a	0 a
	1.0	-	1.0		0 a	100 l	11.1 bc	1.5 ab
	2.0	-	1.0		13.8 b	83.3 jk	0 a	0 a
	4.0	-	1.0		0 a	100 l	0 fa	0 a
	1.0	-	2.0		0 a	86.1 jk	16.6 de	2.0 cb
	1.0	-	4.0		16.6 b	77.7 ij	16.6 de	1.0 ab
	1.0	-	-	1.0	0 a	69.4 hi	0 a	0 a
	2.0	-	-	1.0	0 a	97.2 l	0 a	0 a
	4.0	-	-	1.0	8.3 ab	88.8 jk	0 a	0 a
	1.0	-	-	2.0	0 a	58.3 gh	0 a	0 a
	1.0	-	-	4.0	16.6 b	91.6 k	0 a	0 a
Control					0 a	0 a	0 a	0 a

Values are expressed as means and different letters in the same column represent significantly different values as separated by Tukey's Test ($p \leq 0.05$).

[1]No. of shoots/explant represents the mean number of shoots for responding explant.

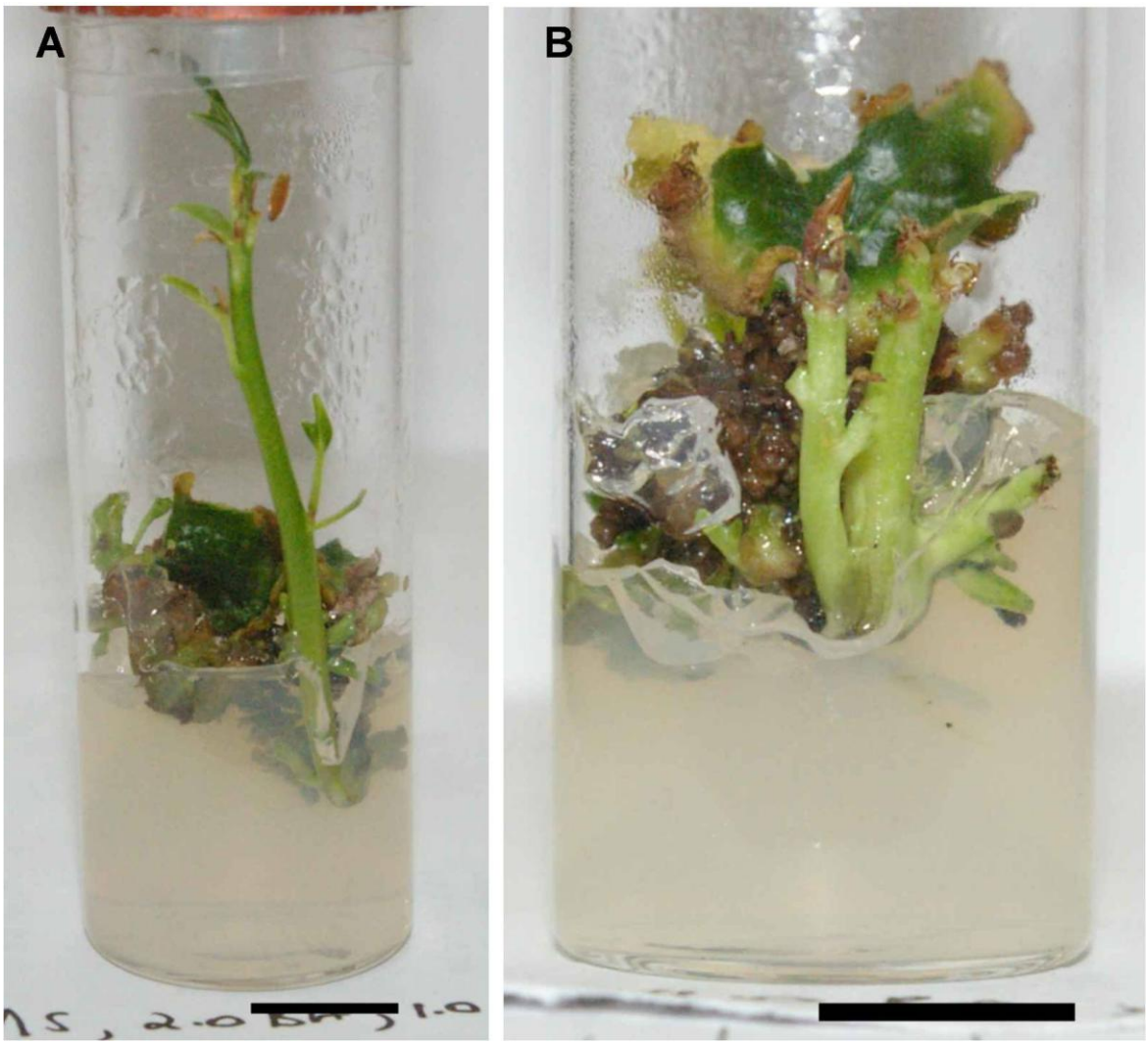


FIGURE 3.2 Plant regeneration from *S. birrea* leaf explants on different concentrations of BA in combination with NAA or IBA. **(A)** A regenerated plantlet on MS supplemented with 2.0 µM BA and 1.0 µM NAA after 8 weeks. **(B)** Plantlets developing on MS, 4.0 µM BA and 1.0 µM NAA after 8 weeks. Scale bar = 10 mm.

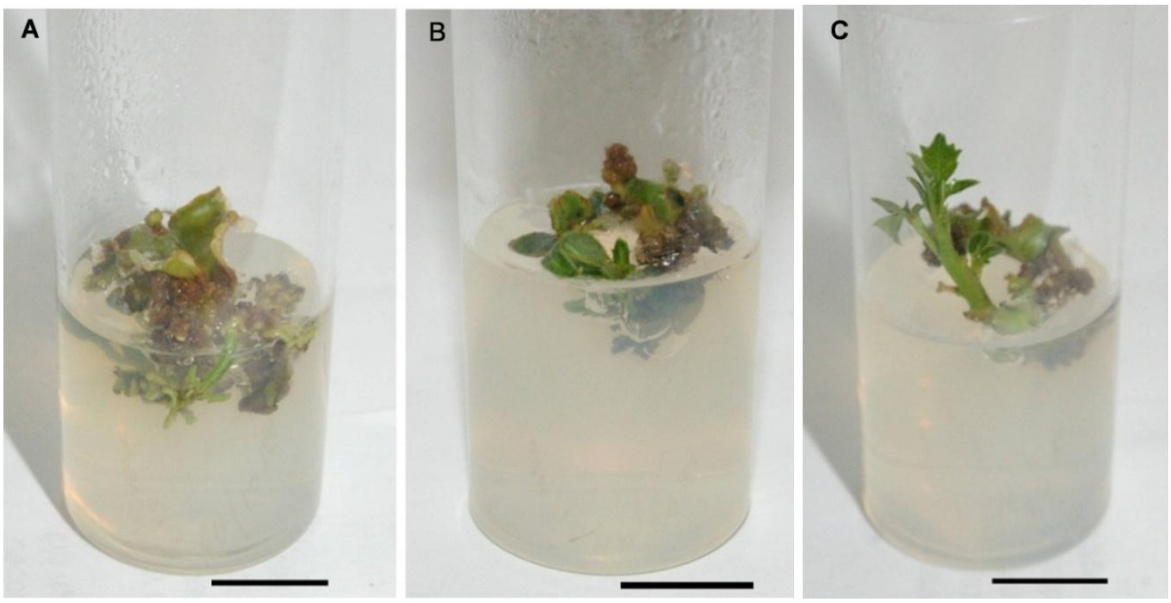


FIGURE 3.3 Plant regeneration from *S. birrea* leaves on WPM supplemented with different concentrations of BA in combination with IBA at 8 weeks. The illustration depicts shoot organogenesis on WPM supplemented with **(A)** 1.0 µM BA and 1.0 µM IBA. **(B)** 1.0 µM BA and 2.0. **(C)** 1.0 µM BA and 4.0 µM IBA. Scale bar = 10 mm.

TABLE 3.2 Shoot regeneration from leaf-derived nodular meristemoids on MS and WPM basal salts in combination with BA and NAA or IBA after 4 weeks in liquid shake culture

Medium	Cytokinin (µM)	Auxin	Differentiated shoots (%)[1]	Mean shoot length (mm) ±SE
	BA	NAA		
MS salts	0	0	25	5.0 ± 0.0b
	2.0	1.0	62	14.3 ± 1.4ab
	4.0	1.0	50	23.5 ± 6.2a
	BA	IBA		
WPM	0	0	50	6.7 ± 1.7b
	1.0	2.0	50	9.0 ± 1.0b

Means ± SE followed by the same letter within the column are not statistically significant using Tukey test ($p \leq 0.05$).

[1]Represents the percentage of explants that differentiated into shoots.

FIGURE 3.4 Direct shoot organogenesis from *S. birrea* leaf-derived nodular meristemoids in liquid shake culture on MS medium supplemented with 2.0 µM BA and 1.0 µM NAA after 8 weeks. Scale bar = 10 mm.

3.2.3 MACROSCOPIC STUDY

Observations using scanning electron microscopy showed that the clusters (Fig. 3.1B) had numerous meristematic buds at different stages of development (Fig. 3.5C). The developing adventitious shoot buds had a defined apical meristem region and several leaf primordia (Fig. 3.5D). The globular structure of the meristemoid clusters was confirmed by histological sections (Fig. 3.6A). The globular structures differentiated into adventitious shoot buds (Fig. 3.6B and C).

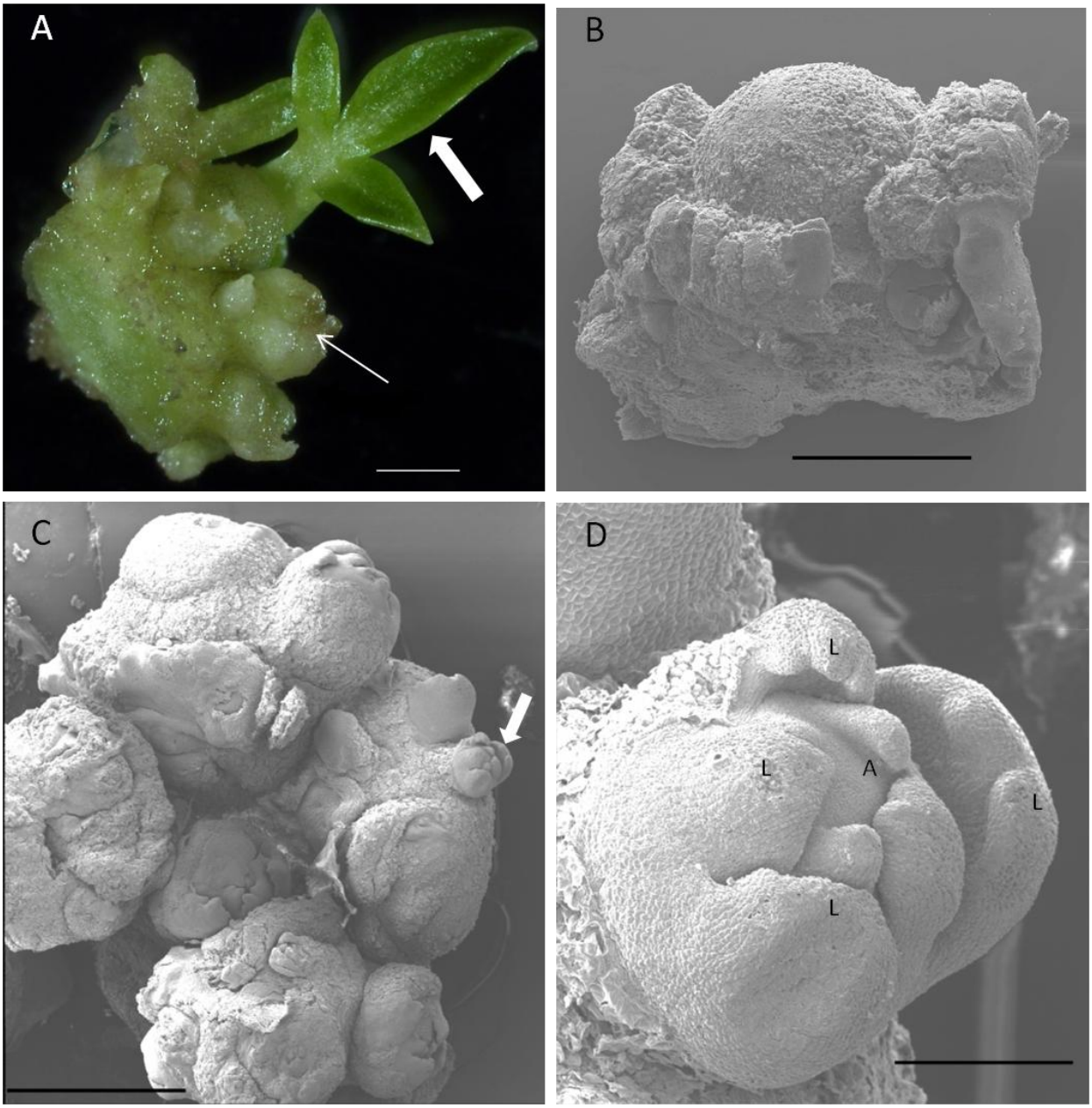


FIGURE 3.5 Initiation of nodular meristemoids and shoot buds on leaf explants of *S. birrea.* **(A)** A stereomicrograph showing chlorophyll containing nodular meristemoid (arrowhead) and a shoot-like structure (large arrow) (Bar = 1.0 mm). **(B)** Scanning electron micrograph of the globular stage nodular meristemoid (Bar = 1.0 mm). **(C)** A scanning electron micrograph showing a cluster of more differentiated shoot buds (Bar = 1.5 mm). The solid arrow points to one of the shoot buds. **(D)** A shoot bud with a defined shoot apical meristem (**A**) and leaf primordia (**L**) (Bar = 430 µm).

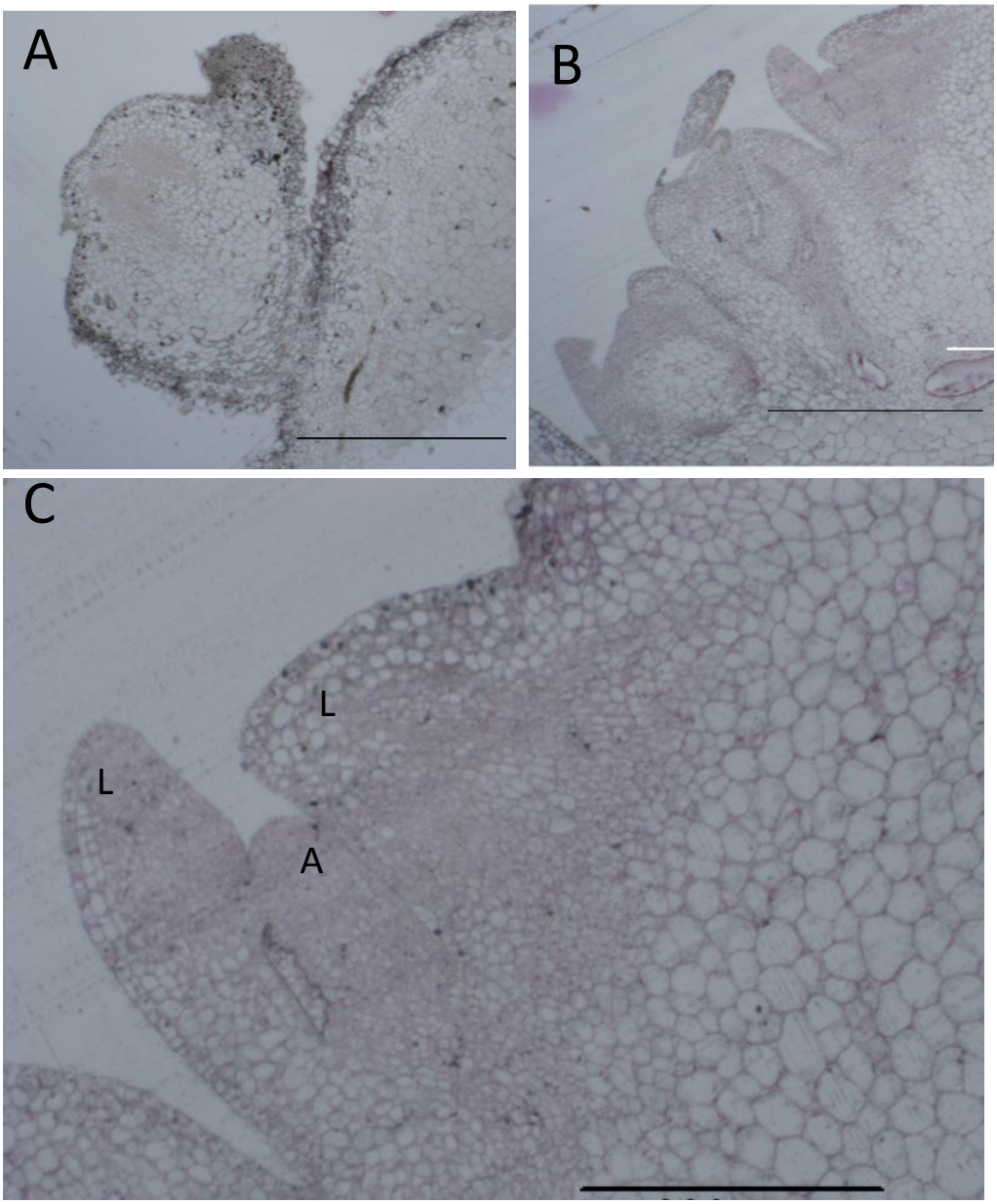


FIGURE 3.6 Histological observations showing the globular and shoot bud stages. **(A)** Globular structure growing from the epidermal cell layers (Bar = 500 µm). **(B)** Longitudinal section of three differentiated buds, complete with enclosing outer primordial leaves (Bar = 500 µm). **(C)** Longitudinal section of at higher magnification bud showing the cytohistological zones associated with the shoot apical meristem (Bar = 200 µm). **A** and **L** indicate the apical meristem and leaf primordia, respectively.

3.3 DISCUSSION

Direct shoot organogenesis was achieved from leaf explants through the induction of nodular meristemoids. Similar structures have been reported as somatic embryo-like structures for *Pelargonium* x *hortorum* Bailey **(HAENSCH 2004)**, *Linum usitatissimum* **(SALAJ et al. 2005)** and *Elliottia racemosa* **(WOO and WETZSTEIN 2008)**. Such structures have, in some cases, been mistaken for true somatic embryos based on their morphological features **(HAENSCH 2004)**. In the present study, nodular meristemoids differentiated directly into shoot apical meristems with distinct cytohistological zonation (Fig. 3.6). Nodular meristemoids have been identified in both direct **(TENG 1997; BATISTA et al. 2000; FERREIRA et al. 2009)**, and indirect morphogenesis pathways through an intervening callus phase **(HICKS 1994; BATISTA et al. 2000; XIE and HONG 2001; TIAN et al. 2008)**. For example, in leaf explants of *Rosa* sp., adventitious shoots were formed from protocorm-like bodies induced from calli **(TIAN et al. 2008)**. The callus produced in the present study did not differentiate into any recognisable organs.

The regenerating meristemoids in the present study exhibited a nodular-shaped structure at the beginning of their development (Fig. 3.5A), with resemblance to globular somatic embryos. However, the distinct lack of bipolarity of these meristematic 'nodules' was confirmed by histological examination (Fig. 3.6). The nodular meristemoids in our study were also connected with a wide base to the surface of the maternal leaf explants (Fig. 3.6). True somatic embryos are bipolar individuals with no vascular connection to the mother plant **(HAENSCH 2004)**. Comparison of regenerating structures by external morphological features (Fig. 3.5A and 3.5B) is insufficient evidence for conclusion of similar embryogenic identity of the formed structures **(HAENSCH 2004; SALAJ et al. 2005)**. The presence of shoot apical meristems with distinct cytohistological zonation (Fig. 3.5D and 3.6C) shows that the regenerants are shoot-like structures.

There was also simultaneous development of numerous chlorophyll-containing nodular meristemoids on single leaf explants (Fig. 3.5C and 3.6B), and sometimes the emergence of secondary nodules. **TENG (1997) and FERREIRA et al. (2009)** also observed that nodules could proliferate into more nodules through budding, and

could be maintained long-term at the nodular developmental phase. These nascent nodular meristemoids exhibited variability in their degree of differentiation on a single explant (Fig. 3.5A). Similar green organogenic nodular clusters were observed on *Humulus lupulus* explants **(BATISTA et al. 2000)**. According to **SCHWARZ et al. (2005)** the high number of meristemoids in different stages of development on an explant is common in *in vitro* morphogenic pathways. The initiation of high numbers of nodular meristemoids per explant provides potential for automated large-scale clonal propagation in bioreactors, *in vitro* phytochemical production and the development of synthetic seed technology, similar to that used in somatic embryogenesis. **McCOWN et al. (1988)** hypothesised that somatic embryogenesis and nodule development were distinct but parallel morphogenic pathways. The article by **McCOWN et al. (1988)** probably provided the first evidence of synthesis of nodular meristemoids as an independent and recognisable organogenic pathway. Since then the body of literature on nodular meristemoids and their significance in organogenesis, especially of tree species, has continued to increase. The present study has shown that plant regeneration through nodular meristemoids provides a viable organogenic pathway. However, the major challenge is the conversion of nodular meristemoids into plantlets. In our study the highest conversion rate was only 22% when meristemoids were maintained on the same medium used for their induction. Furthermore, despite the observed high number of differentiated meristemoids showing distinct cytohistological zones, the highest number of shoots per explant was only 3.5 ± 0.6 on MS supplemented with 4.0 µM BA and 1.0 µM NAA (Table 3.1). The conversion of meristemoids into plantlets was improved to 62% in liquid shake culture, but some of the shoots showed signs of hyperhydration. In *Ananas comosus* about 70% of the nodules regenerated plantlets **(TENG 1997)**, as compared to 22% for *Acacia mangium* **(XIE and HONG 2001)** and 6% for *Cichorium intybus* leaf explants **(PIÉRON et al. 1993)**. One of the major attractive features of nodular meristemoid culture is the ability to maintain nodule status by manipulating the culture medium, thus providing the possibility of synchronisation for mass micropropagation **(TENG 1997)**.

3.4 CONCLUSION

Adventitious shoot regeneration was achieved through the induction of nodular meristemoids from *S. birrea* leaf explants. This regeneration protocol has definite potential for large-scale micropropagation of *S. birrea* and application in molecular plant breeding research programmes. However, further experimentation in an attempt to explain, and improve on the low conversion percentage of meristemoids into plantlets, especially on solid medium, is required.

CHAPTER 4
CAULOGENESIS AND RHIZOGENESIS

4.0 INTRODUCTION

Caulogenesis (adventitious shoot formation) and rhizogenesis (adventitious root induction) are regarded as important phases in developing efficient and reliable *in vitro* propagation systems. Plant growth regulators play an important role in cellular reprogramming during the manipulation of plant tissues *in vitro* for caulogenic and rhizogenic induction. The type and concentration of plant growth regulators used have major influences on the eventual morphogenetic fate of somatic plant cells. To date, cytokinins and auxins constitute the most important groups of plant growth regulators used for inducing *in vitro* organogenesis. **VASIL and HILDEBRANDT (1967)** defined organogenesis as the ability of plant tissues to differentiate into various organs *de novo*. A unique characteristic of plant cells is their ability to dedifferentiate from a particular structural and functional state and develop towards other morphogenetic endpoints depending on the prevailing stimuli, in particular plant growth regulators **(SCHWARZ et al. 2005; GUEYE et al. 2009)**. This developmental plasticity and flexibility of plant cells and tissues allows for the manipulation of their ontogenic programme in culture to achieve predetermined organogenic states. The activation of meristematic centres in plant cells and tissues is highly dependent on environmental and developmental signalling factors **(FEHÉR et al. 2003)**. **GUEYE et al. (2009)** observed that both rhizogenenic induction and callus formation in *Phoenix dactylifera* originated from the same cell types with the developmental endpoint dependent on the concentration of auxin used. The developmental process of organogenesis involves three phases, that is, dedifferentiation, induction and differentiation **(SCHWARZ et al. 2005)**. Dedifferentiation involves the reversion of plant cells to a more flexible developmental phase **(SCHWARZ et al. 2005)**. During this phase, existing translational and transcriptional profiles are altered **(FEHÉR et al. 2003)**, and plant cells are reprogrammed to acquire new fates towards the generation of new organs **(COSTA and SHAW 2007)**. Dedifferentiation facilitates *in vitro* plant cell and tissue manipulation. During the induction phase cells acquire competence and become fully determined for shoot or root primordia initiation **(SCHWARZ et al. 2005)**. In the

differentiation phase of organogenesis, the morphological features of the new organ are clearly discernible **(SCHWARZ et al. 2005)**.

The induction of adventitious roots is an critical, but complex process in *in vitro* propagation that is influenced primarily by the type and concentration of auxin used **(HATZILAZAROU et al. 2006; FOGAÇA and FETT-NETO 2005)**. In the micropropagation of woody plants, rooting is the major limiting factor due to their recalcitrance to *in vitro* manipulation **(FEITO et al. 1996; BALTIERRA et al. 2004; TERESO et al. 2008)**. The rhizogenic developmental process is also influenced by various other factors, for example, temperature, light intensity and light duration, and their interactions. The formation of adventitious roots under the influence of auxins is associated with the process of dedifferentiation, in which predetermined cells switch to a new developmental pathway and produce root primordia **(HATZILAZAROU et al. 2006)**. Whilst auxins are known to promote rooting, the role of cytokinins in rhizogenesis remains controversial, but they are generally regarded as rooting inhibitors **(FEITO et al. 1996)**. On the other hand, numerous reports ascribe a stimulatory role by cytokinins in adventitious shoot formation and growth when used alone or in combination with auxins. When dedifferentiated cells acquire competence for shoot organogenesis the formation of adventitious shoots can be induced by application of exogenous cytokinins **(SUGIYAMA 1999)**. However, due to the lack of biosynthetic and signalling mutants the signal transduction pathway leading to shoot organogenesis under the influence of cytokinins remains to be fully elucidated **(SUGIYAMA 1999; WERNER et al. 2001)**. The objectives of this study were to evaluate the ability of different cytokinins and auxins in the induction of shoots and roots, respectively.

4.1 MATERIALS AND METHODS

4.1.1 CHEMICALS

Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 6-benzyladenine (BA), myo-inositol, vitamins (thiamine HCl, nicotinic acid, pyridoxine HCl and glycine) were obtained from Sigma-Aldrich (St. Louis, USA), agar bacteriological from Oxoid Ltd (Bastingtoke, England), and polyvinlypyrrolidone from BDH Chemicals (Poole, England). Other plant growth regulators, *meta*-topolin [6-(3-

hydroxybenzylamino)purine (*m*T)], *meta*-topolin riboside [6-(-3-hydroxybenzylamino)-9-*β*-*D*-ribofuranosylpurine (*m*TR)], and *meta*-methoxytopolin riboside [6-(-3-methoxybenzylamino)-9-*β*-*D*-ribofuranosylpurine (*mem*TR)] were obtained from Olchemim Ltd, Czech Republic. All other chemicals used to prepare growth media were of analytical grade.

4.1.2 PLANT MATERIAL

Hypocotyls and epicotyls were obtained from 30-60 day-old *S. birrea* seedlings grown in plant growth chambers (Controlled Environments Ltd, Manitoba, Canada) at 25 ± 2 °C under a 16-h photoperiod at a photosynthetic photon flux density of 90-100 µmol m^{-2} s^{-1} provided by cool white fluorescent light (Osram L 58W/640, Germany). Initial shoot explants were obtained from seedlings raised in plant growth chambers, whilst subsequent shoots were from *in vitro* cultured explants. Explants from seedlings raised in plant growth chambers were rinsed thoroughly in running tap water, and surface-decontaminated in 70% alcohol for 1 min followed by 2% sodium hypochlorite with Tween 20 (polyoxyethylene sorbitan monolaurate, Saarchem, Krugersdorp, South Africa) for 20 min. The explants were thoroughly rinsed in sterile distilled water 3 times and cultured on MS growth medium supplemented with vitamins and plant growth regulators.

4.1.3 MEDIA AND GROWTH CONDITIONS

The basal medium consisting of MS supplemented with vitamins, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), polyvinylpyrrolidone (3 g l^{-1}) and plant growth regulators, was used for all the experiments. The medium was adjusted to pH 5.8 with KOH or HCl before adding the gelling agent (8 g l^{-1} Agar Bacteriological, Agar No.1, Oxoid Ltd, Bastingstoke, England) and autoclaving at 121 °C, 15 KPa for 20 min. The culture tubes contained 10 ml of MS medium for all experiments. Cultures were maintained at a temperature of 25 ± 2 °C under either a 16-h or 24-h photoperiod regime. Unless otherwise stated, a constant photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} was provided by cool white fluorescent light (Osram L 58W/640, Germany). Light intensity was measured with a quantum radiation sensor (Model Skp 215, Skye Instruments Ltd, Llandridod Wells, Powys, UK).

4.1.4 INDUCTION OF SHOOT ORGANOGENESIS

Four aromatic cytokinins, BA, *m*T, *m*TR, and *mem*TR at concentrations of 2.0, 4.0 and 8.0 µM were evaluated for the induction of adventitious shoot formation using shoot explants. A set of cultures without any cytokinin served as the control. Each treatment was replicated twice and each replicate consisted of 18 explants. Combined data from the two separate experiments were used for statistical analysis. Data on shoot induction (%), number of adventitious shoots per responding explants, and shoot length were recorded after 4 and 8 weeks in culture under a 16-h photoperiod.

4.1.5 SHOOT INDUCTION FROM HYPOCOTYLS AND EPICOTYLS

Hypocotyl or epicotyl segments (10 mm length) were placed horizontally on MS medium supplemented independently with BA, *m*T, *m*TR, and *mem*TR at different concentrations (1.0, 4.0 and 8.0 µM). The control consisted of hypocotyl or epicotyl segments without any cytokinin supplementation. The treatments consisted of 15 replicates each and the experiment was repeated twice. The data from the two experiments were combined for statistical analysis. The cultures were maintained under a 24-h photoperiod and data on shoot induction (%), number of adventitious shoots per explant, shoot length and shoot fresh weight were collected after 6 weeks.

4.1.6 INDUCTION OF ROOT FORMATION

Adventitious shoots (> 5 mm) were excised from the original explants and transferred to MS medium (as described previously) supplemented with IAA, IBA or NAA at different concentrations (1.0, 2.0, 4.0, 6.0 and 8.0 µM). The control consisted of cultures without auxins. To evaluate the effect of temperature on rhizogenesis, adventitious shoots were cultured on MS medium supplemented with 4.0 µM IBA and maintained at different temperatures (20, 25, 30 and 35 °C) under a 16-h photoperiod. Each treatment was replicated twice and each replicate consisted of 15 explants. The combined data were used for statistical analysis. Data on root induction (%), number of adventitious roots per explant, and root length were recorded after 4 and 8 weeks in culture. The influence of photoperiod on root induction was evaluated on adventitious shoots cultured under 16-h and 24-h

photoperiods, respectively. The shoots were cultured on MS medium supplemented with 4.0 µM IBA, and data collection was done after 8 weeks. The photoperiod experiment was replicated twice and each treatment consisted of 20 explants.

After 8 weeks in culture, rooted plantlets were planted in plastic containers in a 1:1 (v/v) vermiculite and sand mixture and placed in a controlled environment misthouse for four days for acclimation *ex vitro*. A high pressure fog system was used to maintain high relative humidity in a range between 90 and 100%. From the misthouse the plantlets were transferred to a greenhouse in which the temperature was maintained at 25 ± 2 °C.

4.1.7 STOMATAL ANATOMICAL AND MORPHOMETRIC EVALUATION

Leaves were harvested from all treatments during the light period and immediately immersed in cold 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. One disc was cut from each of the four leaves per treatment in a glutaraldehyde solution. Fixation of specimens was done in cold 3% glutaraldehyde in 0.05 M sodium cacodylate buffer and incubated for 24 h at 4 °C as described in section 3.1.5. After the 24 h incubation, the specimens were washed twice for 30 min each in 0.05 M cacodylate, and dehydrated through a graded ethanol series as described in section 3.1.4. The leaf discs were dried in CO_2 in an HCP-2 critical point drier (Hitachi, Japan), mounted on aluminium SEM stubs with the abaxial side up and coated with a 20 nm layer of gold-palladium in an Eiko IB-3 ion coater (Ibaraki, Japan). The specimens were viewed in a ZEISS EVO® MA 15 SEM (Carl Zeiss SMT, Germany) fitted with a digital imaging system driven by SmartSEM software and operating at 10.0 kV and a working distance (WD) of between 10.0-10.5 mm.

For morphometric estimation of stomatal density and size, the methods described by **MIGUENS et al. (1993)** and **SNIDER et al. (2009)** with modifications were used. Four disc areas per leaf were imaged at a magnification of 600X, and the number of mature stomata on each image was counted. Each of these values was treated as n = 1, giving a total of four replicates per treatment for statistical analysis. To compare density, diameter and length of stomata, equi-magnified leaf disc areas (454 µm X 306 µm) of 1.38924 X 10^5 μm^2 each were used, and the number of

stomata was expressed per mm^2. The abaxial surface was used for all the measurements as it has been shown to contain more stomata in other plant species **(MIGUENS et al. 1993; ZACCHINI et al. 1997; MAJADA et al. 2001)**.

4.1.8 EXPERIMENTATION AND DATA ANALYSIS

Data were recorded after 4 and 8 weeks (unless stated otherwise) on the shoot and root multiplication. The experiments were arranged in a completely randomised design and each experiment was repeated twice. Percentage data were arcsin transformed to validate the one-way analysis of variance (ANOVA) assumption of normality of data distribution **(TERESO et al. 2008)** and then subjected to statistical analysis. For all the tested parameters ANOVA was used to find differences among treatments, mean values were separated by Duncan Multiple Range Test and significant effects were accepted for $p \leq 0.05$. Data computations were done using SPSS for Windows (version 15, SPSS®, Chicago).

4.2 RESULTS AND DISCUSSION

4.2.1 SHOOT ORGANOGENESIS UNDER THE INFLUENCE OF CYTOKININS

Moderately high adventitious shoot formation from 62.7% of shoot explants was achieved at the highest tested concentration of 8.0 µM *m*T after 8 weeks in culture (Table 4.1). The greatest number of adventitious shoots per explant (2.4 ± 0.30) was also recorded at a concentration of 8.0 µM (Table 4.1). The longest shoots were measured in the 8.0 µM *m*T (23.5 ± 3.16 mm), 4.0 µM BA (22.7 ± 4.29 mm), 4.0 µM *m*T (19.7 ± 2.45 mm) and 2.0 µM BA (19.2 ± 2.76 mm) treatments, respectively. At equimolar concentrations, *m*T and *m*TR produced qualitatively better shoots (Fig. 4.1). The lowest adventitious shoot regeneration capacity was observed in *mem*TR treatments (Table 4.1 and Fig. 4.1B). **PULLMAN and TIMMIS (1992)** obtained a mean multiplication factor of 2.2 from shoot explants of *Pseudotsuga menziesii*. Most of the cytokinin treatments produced basal callus which progressively turned brown over the duration of the culture period (Fig. 4.1 and 4.2). The observed basal callus is a potential sink for cytokinin conjugates that are inhibitory to further proliferation of adventitious shoots. In particular, exogenous cytokinins in plant tissue cultures can be converted to the irreversible N^7- and N^9-glucoside conjugates, which are biologically inhibitory and cannot be hydrolysed to the active free base form **(VAN**

STADEN and CROUCH 1996; AUER 1997). WERBROUCK et al. (1995) detected high quantities of 9-glucosides from BA treated *Spathiphyllum floribundum* plants at the basal sections, unlike the free base-BA and its riboside derivatives which also occurred in other plant parts. The 9-glucosides persisted in the plant tissues and could still be detected nine weeks after transfer of the plants *ex vitro* **(WERBROUCK et al. 1995)**. On the other hand, the free base-BA and its riboside derivatives (9R-BA), originally found in low amounts *in vitro*, could no longer detected one week after transfer *ex vitro* **(WERBROUCK et al. 1995)**. Recent work in our laboratory on *Harpagophytum procumbens* has shown that comparatively higher levels of 9-glucosides accumulate in the basal sections of shoot explants especially in BA-treated plants **(BAIRU per. comm.)**. *Meta*-topolin treated explants had lower levels of 9-glucosides and elevated levels of *O*-glucosides ascribed to the presence a hydroxyl group on its benzyl ring **(BAIRU per. comm.)**. *O*-glucosides are storage forms of cytokinins (*meta*- and *ortho*-topolins), which act as reservoirs for the free base forms through the hydrolysis action of *β*-glucosidase **(STRNAD 1997)**. At equimolar concentrations larger adventitious shoots were produced with *meta*-topolin and *meta*-topolin riboside compared to benzyladenine, suggesting that the levels of the free base and *O*-glucosides were higher in the topolin cytokinins (Fig. 4.1). *In vitro* shoot proliferation is not easily achieved for woody species exhibiting episodic growth patterns due to predetermined developmental cues that are inherent in plant tissues **(McCOWN 2000)**. **VIEITEZ et al. (2009)** observed very slow *in vitro* shoot growth due to the episodic growth character of the woody species, *Quercus alba, Quercus bicolor* and *Quercus rubra*. The episodic nature of *S. birrea* growth could have had a confounding effect on shoot proliferation.

TABLE 4.1 Cytokinin type and concentration effects on *S. birrea* shoot organogenesis at 4 and 8 weeks in culture

Type of cytokinin	Conc. (µM)	4 weeks		8 weeks		
		Adventitious shoots (%)[1]	Mean no. of shoots/explant	Adventitious shoots (%)[1]	Mean no. of shoots/explant	Mean shoot length (mm)
BA	2.0	53.0 ± 6.92 cd	1.8 ± 0.40 b	56.9 ± 3.07 cd	1.7 ± 0.20 cd	19.2 ± 2.76 d
	4.0	51.2 ± 11.25 cd	1.2 ± 0.13 b	51.2 ± 11.25 cd	1.3 ± 0.15 bcd	22.7 ± 4.29 d
	8.0	43.3 ± 3.33 bcd	1.3 ± 0.21 b	46.6 ± 0.00b cd	1.3 ± 0.13 bc	18.5 ± 4.84 cd
*m*T	2.0	50.5 ± 9.41 bcd	2.0 ± 0.48 b	59.1 ± 5.83 cd	1.9 ± 0.23 cd	16.8 ± 2.82 cd
	4.0	39.7 ± 6.41 bcd	1.2 ± 0.18 b	43.5 ± 10.25 bcd	1.8 ± 0.27 cd	19.7 ± 2.45 d
	8.0	62.7 ± 3.92 d	1.7 ± 0.38 b	62.7 ± 3.92 d	2.4 ± 0.30 d	23.5 ± 3.16 d
*m*TR	2.0	40.3 ± 7.01 bcd	1.6 ± 0.23 b	58.1 ± 0.21 cd	1.7 ± 0.20 cd	13.8 ± 2.73 bcd
	4.0	41.9 ± 16.91 bcd	1.4 ± 0.22 b	44.8 ± 19.85 bcd	1.4 ± 0.21 bcd	14.0 ± 3.35 bcd
	8.0	20.0 ± 20.13 abc	1.1 ± 0.12 b	28.9 ± 17.19 abc	1.3 ± 0.18 bcd	15.8 ± 3.00 cd
*mem*TR	2.0	2.5 ± 2.50 a	0.2 ± 0.20 a	2.5 ± 2.50 a	0.5 ± 0.50 a	2.5 ± 2.50 ab
	4.0	13.7 ± 8.47 ab	1.4 ± 0.40 b	16.5 ± 11.25 ab	1.6 ± 0.60 cd	6.2 ± 1.25 abc
	8.0	23.8 ± 6.18 abc	1.3 ± 0.33 b	28.8 ± 4.57 abc	1.4 ± 0.20 bcd	11.6 ± 6.66 abcd
Control		0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a

[1]Represents the percentage of explants producing adventitious shoots.

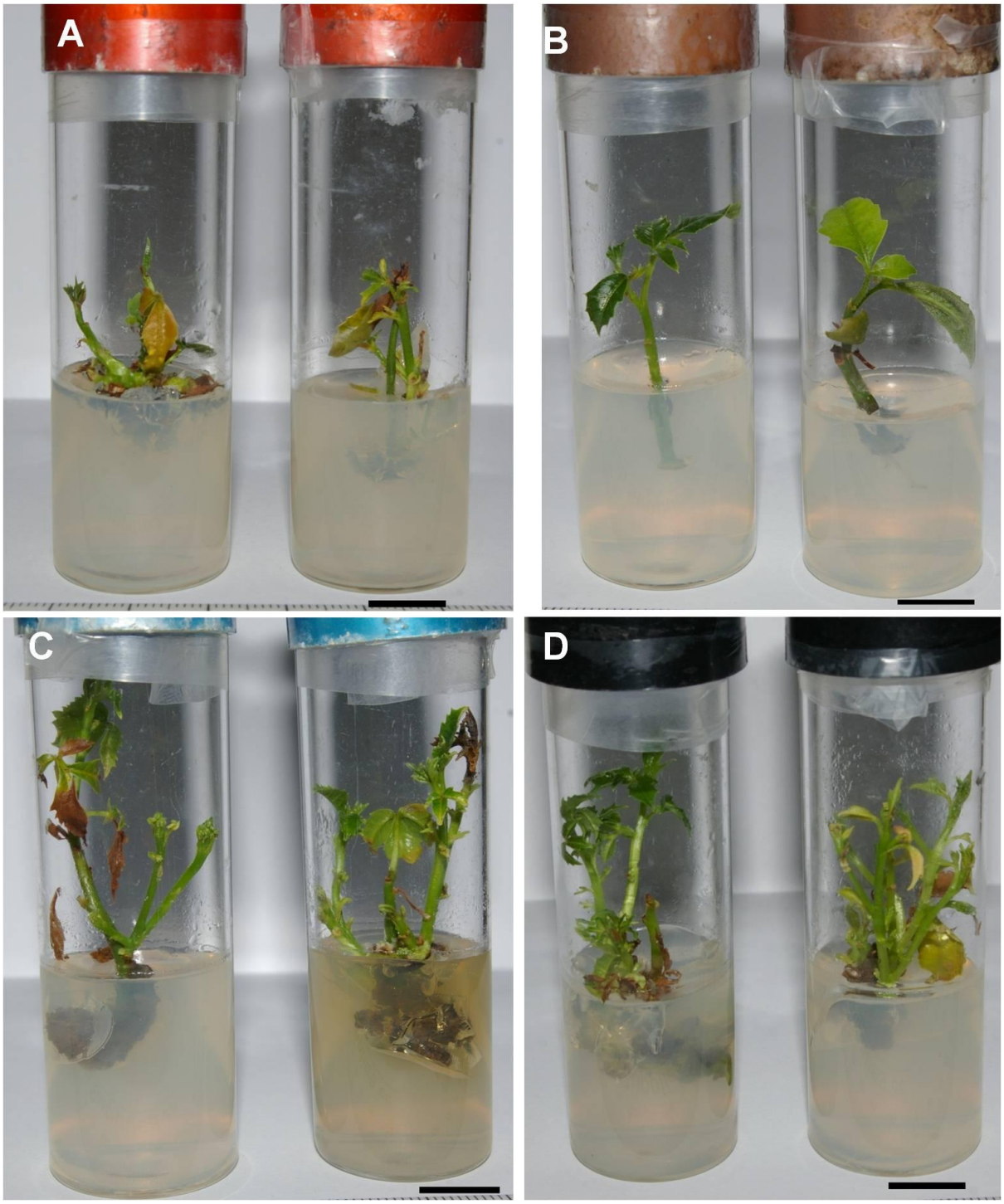


FIGURE 4.1 Effects of aromatic cytokinins on caulogenic induction in *S. birrea* shoot explants after 8 weeks in culture. **(A)** BA (2.0 µM). **(B)** *mem*TR (2.0 µM). **(C)** *m*T (2.0 µM). **(D)** *m*TR (2.0 µM). Scale bar = 10 mm.

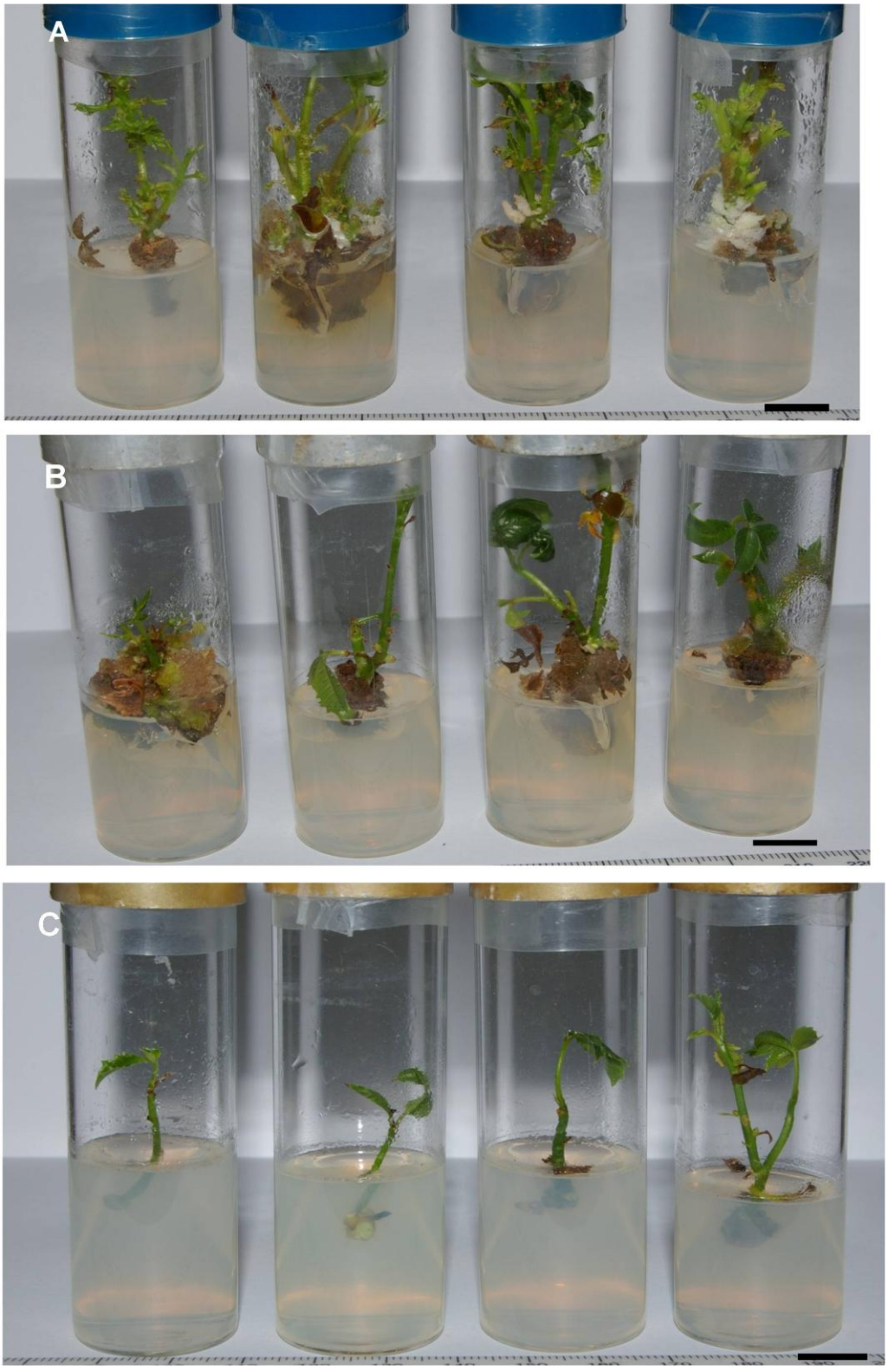


FIGURE 4.2 Effects of aromatic cytokinins on caulogenic induction in *S. birrea* shoot explants after 8 weeks in culture. **(A)** *m*T (8.0 µM). **(B)** BA (8.0 µM). **(C)** *mem*TR (8.0 µM). Scale bar = 10 mm.

4.2.2 SHOOT ORGANOGENESIS FROM HYPOCOTYLS AND EPICOTYLS

The *in vitro* cultured hypocotyl segments on medium containing cytokinins enlarged and produced swollen nodular-like shaped tissue at the distal cut ends during the first two to three weeks (Fig. 4.3). The distal cut end represents the section of the hypocotyl explant that would be furthest from the shoot apex on an intact seedling. This growth indicates a high level of cell division due to the presence and active uptake of cytokinins from the initiation medium. Cytokinins are known to play a central role at the cellular level by regulating the cell cycle **(WERNER et al. 2001)**. As similarly observed for *Cuminum cyminum* explants by **TOWFIK and NOGA (2001)** the swollen tissues were compact, nodular and dark green (Fig. 4.3). Within a period of two to three weeks the first shoot primordia were observed using a stereomicroscope (Fig. 4.4). Similar to findings in this study, **DE PAIVA NETO et al. (2003)** observed direct shoot organogenesis along the distal ends of hypocotyl explants of *Bixa orellana*. Hypocotyls were highly sensitive to the concentration of BA in the medium. At a concentration of 1.0 µM BA, shoot primordia were clearly discernible on the distal ends of hypocotyl explants two to three weeks after culture initiation (Fig. 4.4A). On the other hand, high concentrations of BA (4.0 µM) induced callus formation on the explants (Fig. 4.4B). Induction of shoot organogenesis on the distal ends could be influenced by an auxin-gradient and auxin/cytokinin balance resulting from polar auxin transport **(DE PAIVA NETO 2003)**. The highest adventitious shoot formation occurred in 55% of hypocotyl explants on MS medium supplemented with 8.0 µM *m*T (Table 4.2). A concentration of 4.0 µM *m*TR induced shoot formation in 49.1% of hypocotyl explants (Table 4.2). The highest shoot induction (20.8%) on BA-supplemented medium was observed at a concentration of 1.0 µM (Table 4.2). Higher BA concentrations lead to callus formation which did not differentiate into shoots or any recognisable organs (Fig 4.4B). In *Bixa orellana* hypocotyls, **DE PAIVA NETO et al. (2003)** recorded the highest shoot induction response of 81.7% on initiation medium with thidiazuron, while **MAKUNGA and VAN STADEN (2008)** observed direct shoot organogenesis from 52% of *Salvia africana-lutea* hypocotyl explants on basal medium supplemented with IAA and kinetin. In the present study, the longest shoots (31.2 ± 3.08 mm) were obtained on initiation medium with 8.0 µM *m*T (Table 4.1 and Fig. 4.5A). The same concentration of *m*T resulted in the highest fresh weight shoot of 118 ± 20.50 mg. Fresh weight was also

relatively high for 8.0 µM *m*TR (69.7 ± 10.11 mg) (Table 4.2 and Fig. 4.5B). Shoot induction at 8.0 µM *m*TR was 40%.

Compared to hypocotyls, caulogenesis was lower for epicotyl explants across the tested equimolar concentrations of the aromatic cytokinins, BA, *m*T, *m*TR and *mem*TR (Table 4.3). These results conform to the observations by **SHARMA AND RAJAM (1995); DE PAIVA NETO (2003)** and **MAKUNGA AND VAN STADEN (2008)** that different explants may have different morphogenic capacities. The highest shoot induction from 25.7% of epicotyls explants was obtained on MS medium supplemented with 8.0 µM *m*TR. The highest number of adventitious shoots per explant (2.1 ± 0.60) was induced on 4.0 µM *m*TR.

TABLE 4.2 Shoot induction in *S. birrea* hypocotyls after 6 weeks in culture under different cytokinin treatments

Type of cytokinin	Conc. (µM)	% of explants producing shoots	No. of shoots/ explant	Shoot length (mm)	Shoot fresh mass (mg)
BA	1.0	20.8 ± 4.16 abcd	1.0 ± 0.00 b	12.6 ± 3.54 b	19.8 ± 7.43 ab
	4.0	10.0 ± 10.0 ab	1.0 ± 0.00 b	15.0 ± 5.00 b	19.5 ± 0.50 ab
	8.0	17.8 ± 17.8 abcd	1.8 ± 0.37 c	15.8 ± 1.92 b	30.1 ± 4.47 ab
*m*T	1.0	30.0 ± 10.0 bcdef	1.0 ± 0.00 b	15.5 ± 2.60 b	39.0 ± 6.68 ab
	4.0	41.6 ± 8.33 def	1.0 ± 0.00 b	21.2 ± 7.42 bc	63.7 ± 22.01 b
	8.0	55.0 ± 5.00 f	1.6 ± 0.28 bc	31.2 ± 3.08 c	118.8 ± 20.50 c
*m*TR	1.0	25.0 ± 8.33 abcde	1.1 ± 0.16 bc	13.7 ± 2.64 b	25.4 ± 8.17 ab
	4.0	49.1 ± 4.70 ef	1.6 ± 0.27 bc	16.3 ± 3.46 b	40.7 ± 6.30 ab
	8.0	40.0 ± 1.60 cdef	1.1 ± 0.10 bc	17.5 ± 2.64 b	69.7 ± 10.11 b
*mem*TR	1.0	13.3 ± 3.33 abc	1.0 ± 0.00 b	11.6 ± 1.66 ab	24.6 ± 11.66 ab
	4.0	29.2 ± 9.23 bcde	1.0 ± 0.00 b	11.5 ± 1.10 ab	31.4 ± 5.08 ab
	8.0	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a
Control		0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a

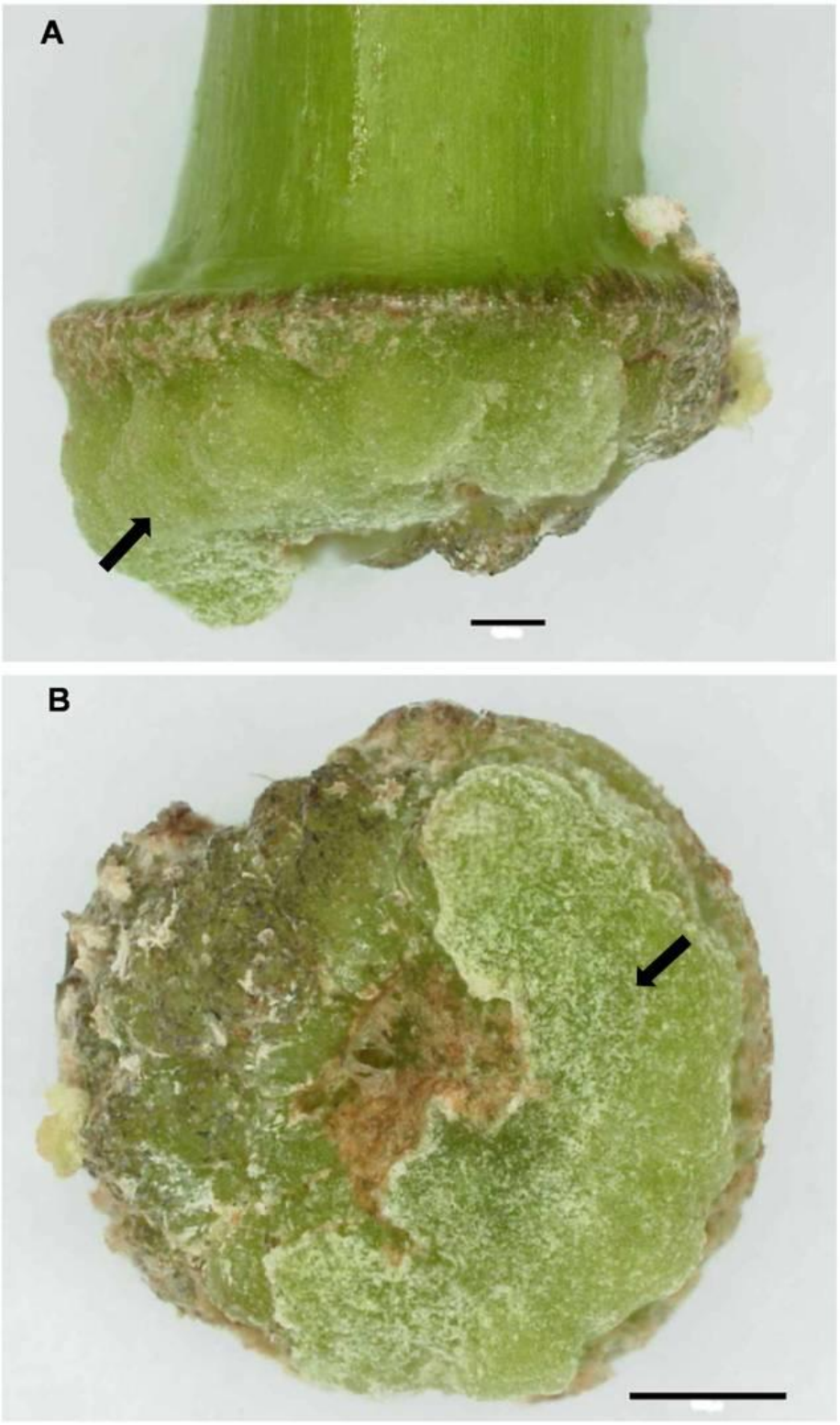


FIGURE 4.3 Stereomicrographs showing the swollen nodular-like tissue (solid arrows) developing on the distal ends of hypocotyls in the presence of a cytokinin stimulus during the first two to three weeks in culture. Scale bars represent **(A)** 1.0 mm and **(B)** 2.0 mm.

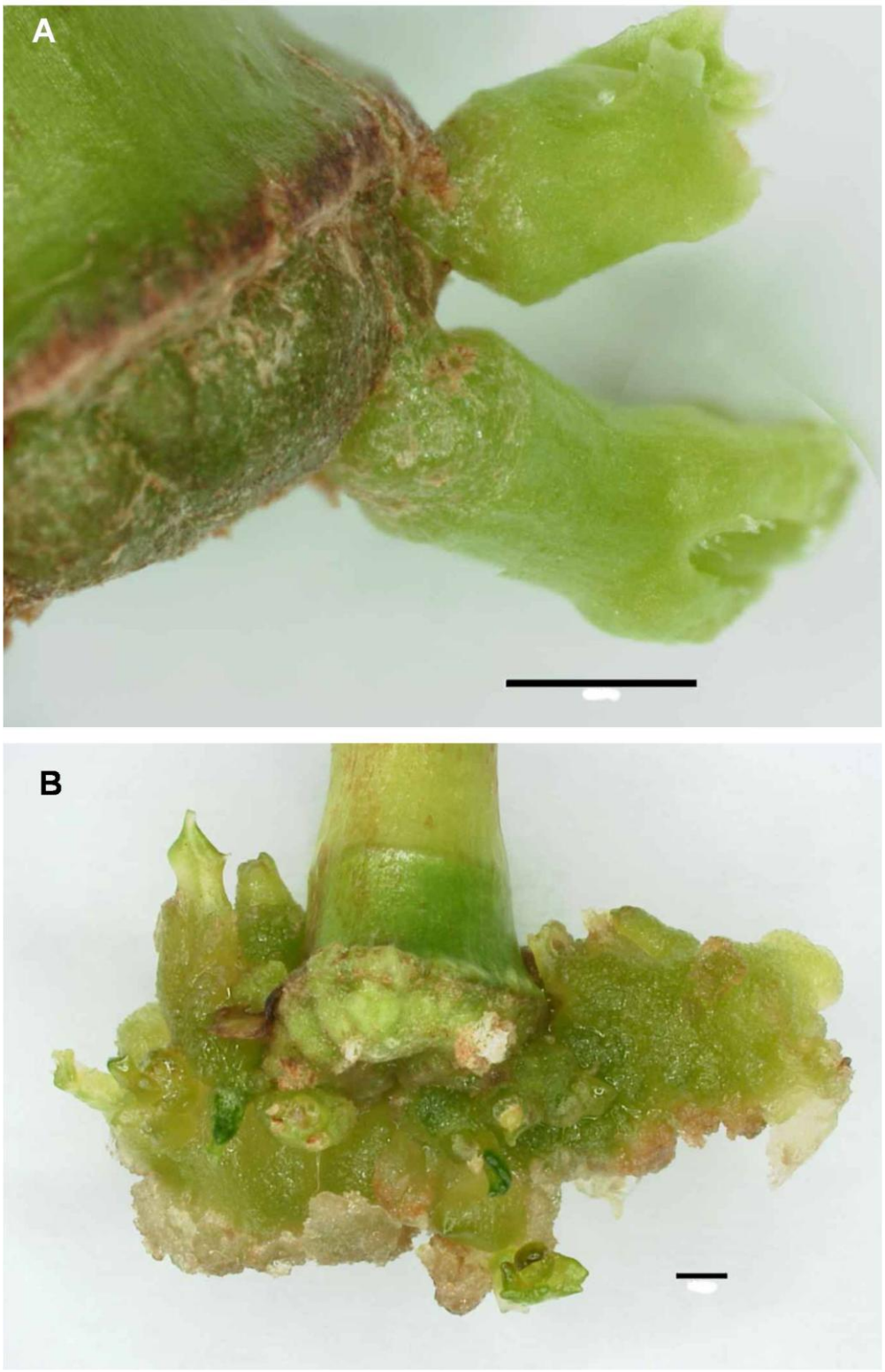


FIGURE 4.4 Effects of benzyladenine on caulogenic induction in *S. birrea* hypocotyl explants after 3 weeks in culture. **(A)** Shoot buds forming on 1.0 µM BA (Scale bar = 2.0 mm) and **(B)** Callus formation on 4.0 µM BA (Scale bar = 1.0 mm).

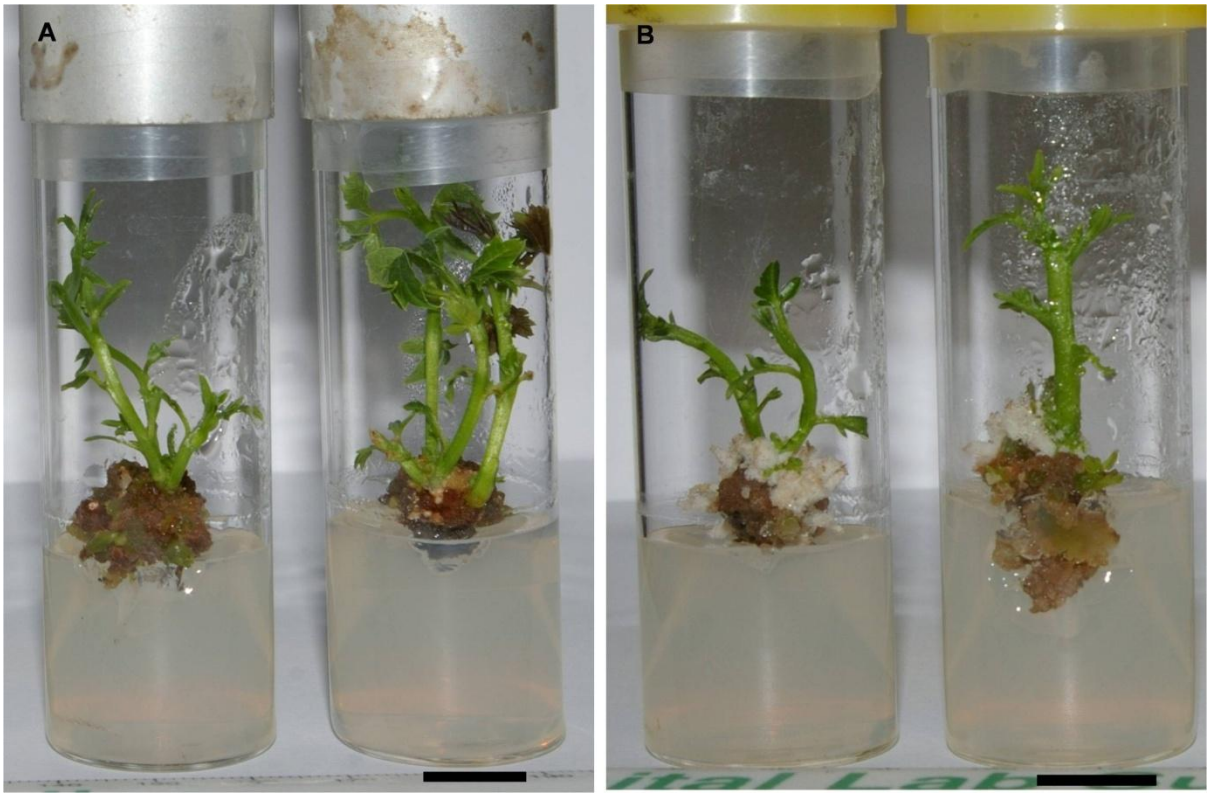


FIGURE 4.5 Shoot regeneration from *S. birrea* hypocotyls explants after 6 weeks in culture. **(A)** *m*T (8.0 µM). **(B)** *mem*TR (8.0 µM). Scale bar = 10 mm.

TABLE 4.3 Induction of shoots in *S. birrea* epicotyls after 6 weeks in culture under different cytokinin treatments

Type of cytokinin	Conc. (µM)	% of epicotyls producing shoots	No. of shoots/ explant
BA	1.0	4.5 ± 4.54 ab	1.5 ± 0.50 bc
	4.0	5.9 ± 0.70 abc	2.0 ± 1.00 bc
	8.0	0.0 ± 0.00 a	0.0 ± 0.00 a
*m*T	1.0	8.0 ± 1.42 abc	1.0 ± 0.00 ab
	4.0	14.6 ± 8.03 abcd	1.0 ± 0.00 ab
	8.0	17.5 ± 2.50 bcd	1.1 ± 0.16 bc
*m*TR	1.0	20.3 ± 6.96 cd	1.0 ± 0.00 ab
	4.0	15.2 ± 8.57 abcd	2.1 ± 0.60 c
	8.0	25.7 ± 5.78 d	1.2 ± 0.14 bc
*mem*TR	1.0	14.1 ± 5.83 abcd	1.0 ± 0.00 ab
	4.0	3.3 ± 3.33 ab	1.0 ± 0.00 ab
	8.0	7.6 ± 1.01 abc	1.3 ± 0.33 bc
Control		0.0 ± 0.00 a	0.0 ± 0.00 a

4.2.3 RHIZOGENIC INDUCTION IN SHOOTS

Table 4.4 presents the results of rooting experiments after 8 weeks in culture. Treatment of shoots with three different auxins at concentrations of 1.0, 2.0, 4.0, 6.0 and 8.0 µM resulted in varied rooting responses. Fifty percent rhizogenic induction was observed on auxin-free cultures, suggesting the presence of endogenous auxins in the explants. The highest rooting, 91.6% and 83.3% after 8 weeks in culture, was achieved with IBA-treated shoots, at concentrations of 4.0 and 6.0 µM, respectively. The lowest rooting response for IBA-treatments (25%) was observed for the highest tested concentration of 8.0 µM. At least 50% root induction was obtained for IBA treatments in the range 1.0-6.0 µM, but beyond 4.0 µM the roots were stunted

(Fig. 4.6). The highest root induction response for IAA-treated and NAA-treated shoot explants after 8 weeks in culture was 58.3% obtained on 2.0 and 4.0 µM, respectively. Across the evaluated auxins and different concentrations, the lowest rhizogenic induction was observed on MS medium supplemented with 6.0 µM IAA (8.3%) and 8.0 µM NAA (16.6%). The number of roots per rooting explant was highest for 4.0 µM IBA (5.4 ± 1.18) and 6.0 µM (4.2 ± 1.04). Auxin-free medium and cultures treated with 4.0 µM IBA had the longest roots measuring 30 ± 9.96 mm and 26 ± 6.64 mm, respectively.

DE KLERK et al. (1999); **EPSTEIN and LUDWIG-MÜLLER (1993)**; and **FOGAÇA and FETT-NETO (2005)** reported that IBA, the widely used auxin in commercial operations, was stable and less prone to photo-oxidation compared to IAA. In the present study, IBA produced consistently high rooting (≥ 50%) over a wider concentration range of 1.0 to 6.0 µM, compared to both IAA and NAA. At higher concentrations NAA exhibited low root induction capacity. **DE KLERK et al. (1995)** observed that beyond a certain optimum level, higher concentrations of auxins may be inhibitory to rhizogenesis. The rooting explants in this study also produced lateral roots (data not shown). **EPSTEIN and LUDWIG-MÜLLER (1993)** attributed the superior rooting ability of IBA compared to IAA to the higher stability of IBA and differences in their metabolism and transport. Indole-3-butyric acid may be a simple 'conjugate' of IAA and is converted to IAA by *β*-oxidation **(LUDWIG-MÜLLER et al. 2005)**. New emerging evidence further suggests that part of the effects of IBA are caused solely by its direct action **(LUDWIG-MÜLLER et al. 2005)**, and this may partially account for the differences in rooting abilities observed in this study. **TERESO et al. (2008)** attributed the differential rooting abilities of auxins to different affinity of each auxin to the auxin receptors involved in the rhizogenic process.

The highest rooting in the presence of 4.0 µM IBA was induced at a temperature of 25 °C (Table 4.5). After 8 weeks in culture at this temperature, 83.3% of the shoots produced roots with an average of 4.5 ± 1.18 roots per shoot. This was lower than the average for the previous experiment, suggesting that inherent episodic factors were affecting growth. **NORTON and NORTON (1986)** and **NAIK et al. (2003)** also observed a systematic decline in shoot and root formation over successive sub-

cultures. There was no significance difference in rooting between shoots grown under 16-h and 24-h photoperiods (Table 4.6). However, rooting was marginally higher under the 16-h photoperiod, but there was wide variation in response over the two replicates. When *in vitro* rooted shoots were transferred to the *ex vitro* environment they survived for only two weeks. Success in *in vitro* rooting facilitates acclimation by improving water uptake **(DE KLERK 2002)**. However, the difficulties of *in vitro* rooting and acclimation of woody species has been widely reported **(PULLMAN and TIMMIS 1992)**. Working on *Pseudotsuga menziesii*, **PULLMAN and TIMMIS (1992)** achieved *ex vitro* rooting and soil establishment success of 5-17% depending on the genotype, and this increased to 8-60% when the *in vitro* culture period was prolonged by one to two years. **TERESO et al. (1998)** reported on limited acclimation success as only a few *in vitro* rooted *Prunus dulcis* plantlets survived the *ex vitro* environment. In *Pistacia vera* 80% *in vitro* rooting was achieved using 2.0 mg l^{-1} IBA, and 80% of the plantlets survived *ex vitro* **(TILKAT and ONAY 2009)**. The *in vitro* rooting success in the present study was 91% using IBA, but some of the roots were still brittle after 8 weeks, and may have matured if afforded a more prolonged culture period. A longer *in vitro* rooting procedure may allow for further development of more hardy roots with a high chance of surviving the *ex vitro* environment.

TABLE 4.4 Rhizogenic effects of IBA, IAA and NAA on *S. birrea* shoot explants after 4 and 8 weeks in culture

Type of Auxin	Conc. (µM)	4 weeks		8 weeks		
		% of rooting explants	Mean no. of roots/explant	% of rooting explants	Mean no. of roots/explant	Mean root length (mm)
Control	0.0	50.0 ± 14.43 abc	0.9 ± 0.31 abc	50.0 ± 14.43 a	1.1 ± 0.44 a	30.0 ± 9.96 c
IBA	1.0	41.6 ± 16.66 abc	1.5 ± 0.71 abc	50.0 ± 12.50 ab	1.7 ± 0. 66 ab	15.6 ± 6.24 abc
	2.0	50.0 ± 25.00 abc	2.4 ± 1.09 bc	58.3 ± 14.43 ab	2.5 ± 1.00 ab	8.3 ± 2.97 ab
	4.0	83.3 ± 16.66 c	4.3 ± 0.97 d	91.6 ± 7.21 c	5.4 ± 1.18 c	26.0 ± 6.64 bc
	6.0	58.3 ± 8.33 bc	2.8 ± 0.77 cd	83.3 ± 7.21 bc	4.2 ± 1.04 bc	16.6 ± 5.82 abc
	8.0	16.6 ± 16.66 ab	0.4 ± 0.36 ab	25.0 ± 14.43 a	0.9 ± 0.60 a	11.0 ± 7.13 abc
IAA	1.0	58.3 ± 22.04 bc	1.5 ± 0.70 abc	50.0 ± 14.43 ab	2.4 ± 1.30 ab	14.7 ± 6.47 abc
	2.0	25.0± 14.43 ab	1.0 ± 0.63 abc	58.3 ± 8.33 ab	2.4 ± 0.87 ab	10.2 ± 4.30 ab
	4.0	25.0 ± 14.43 ab	0.7 ± 0.46 ab	41.6 ± 16.66 a	2.0 ± 0.90 ab	13.8 ± 5.76 abc
	6.0	0.0 ± 0.00 a	0.0 ±0.00 a	8.3 ± 8.33 a	0.1 ± 0.18 a	7.0 ± 7.09 ab
	8.0	16.6 ± 8.33 ab	0.8 ± 0.55 abc	25.0 ± 0.00 a	1.8 ± 1.03 ab	7.6 ± 4.47 ab
NAA	1.0	25.0 ± 14.43 ab	0.4 ± 0.24 ab	33.3 ± 8.33 a	0.7 ± 0.33 a	1.4 ± 0.65 a
	2.0	33.3 ± 8.33 ab	1.0 ± 0.44 abc	33.3 ± 8.33 a	1.1 ± 0.53 a	2.6 ± 1.80 a
	4.0	16.6 ± 8.33 ab	0.7 ± 0.42 ab	58.3 ± 8.33 ab	2.4 ± 0.74 ab	15.9 ± 6.29 abc
	6.0	25.0 ±14.43 ab	1.1± 0.72 abc	41.6 ± 30.04 ab	1.6 ± 0.80 ab	12.1 ± 6.30 abc
	8.0	0.0 ± 0.00 a	0.0 ± 0.00 a	16.6 ± 8.33 a	0.3 ± 0.27 a	0.9 ± 0.60 a

[a]Mean root length is calculated from the longest root recorded per treatment.

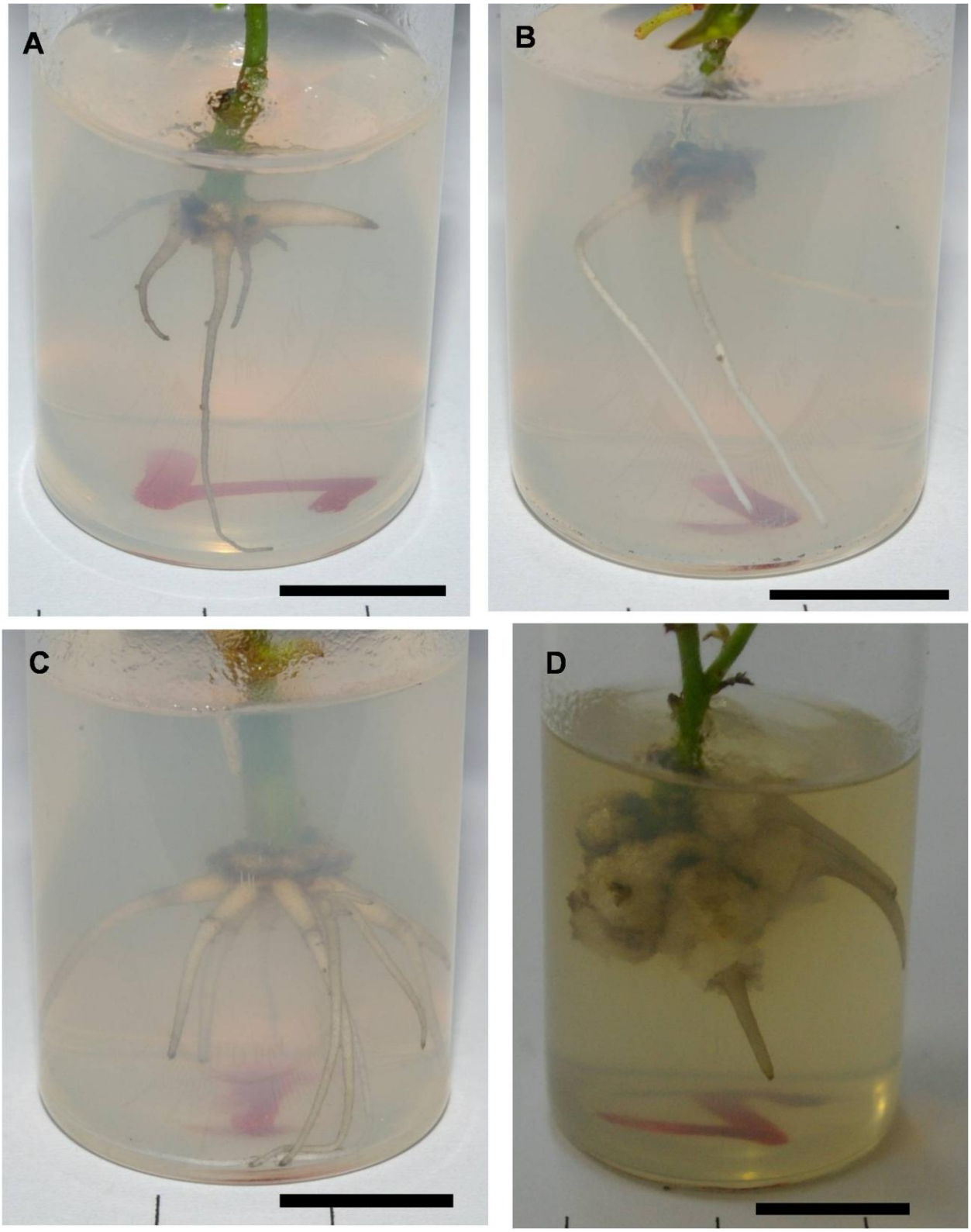


FIGURE 4.6 Rhizogenic induction under the influence of increasing concentrations of IBA in *S. birrea* shoots after 8 weeks in culture. **(A)** 1.0 µM. **(B)** 2.0 µM. **(C)** 4.0 µM. **(D)** 6.0 µM. Scale bar = 10 mm.

TABLE 4.5 Temperature effects on rooting of *S. birrea* shoot explants cultured on 4.0 µM IBA after 4 and 8 weeks

Temperature (°C)	4 weeks		8 weeks		
	% of rooting explants	Mean no. of roots/explant	% of rooting explants	Mean no. of roots/explant	Mean root length (mm)
20	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 a
25	58.3 ± 8.33 b	2.5 ± 0.86 b	83.3± 16.66 c	4.5 ± 1.18 c	21.3± 6.64 b
30	25.0 ± 14.43 a	0.8 ± 0.46 a	66.6± 8.33 bc	2.6 ± 0.73 bc	17.7± 9.30 ab
35	25.0 ± 14.43 a	1.1 ± 0.90 ab	33.3± 8.33 ab	1.7 ± 1.03 ab	19.2± 9.60 ab
40	0.0 ± 0.00 a	0.0 ± 0.00 a	8.3± 8.33 a	0.08 ± 0.08 a	0.4± 0.41 a

Data represent mean ± SE.

Different letters in the same column represent significantly different values as separated by Duncan Multiple Range Test.

Mean root length represents the mean of the longest root in each treatment.

TABLE 4.6 Effect of 16-h and 24-h photoperiods on rooting

Treatment	% root induction	No. of roots/ explant
16-h light/ 8-h dark	43.4 ± 19.67	3.7 ± 0.59
24-h light	30.0 ± 5.00	4.2 ± 1.14
F probability	0.475	0.166
Significance (*t*-test)	NS	NS

NS denotes not significant at $p \leq 0.05$.

4.2.4 PHOTOPERIODIC EFFECTS ON STOMATAL DENSITY AND MORPHOLOGY

Results of stomatal density and dimensions are presented in Table 4.7. Stomatal density (number of stomata per mm^2) on the abaxial leaf surfaces was highest for the control plants propagated from seeds (221.9 ± 13.99) (Fig. 4.9) and the 16-h photoperiod treatment (206.6 ± 15.28) (Fig. 4.7). Stomata were longest (18.4 ± 0.47 µm) for the 24-h light treatment (Table 4.7 and Fig. 4.8) and the control (18.3 ± 0.39 µm) (Table 4.7 and Fig. 4.9). Stomatal width was also greatest for plants grown under the 24-h photoperiod (17.7 ± 0.37 µm). The results indicate an inverse relationship between density and size of stomata. Mature stomata have a pore that is surrounded by two kidney-shaped guard cells and may be normal or abnormal **(MIGUENS et al. 1993)**. Normal stomata have kidney-shaped guard cells with an outer ledge over the stomatal pore **(MIGUENS et al. 1993)**. Stomata of plants grown in the control (Fig. 4.10C) and 16-h photoperiod treatment (Fig. 4.10A) fit this description of mature stomata. Fig. 4.10 depicts the morphology of typical stomata under the three treatments. Stomata from the 16-h photoperiod plants have fully expanded guard cells around the pore (Fig. 4.10A) and they closely resemble stomata in the control (Fig. 4.10C). Abnormal stomata of leaves grown under a 24-h photoperiod had the ellipsoidal shape, but a poorly developed pore and external periclinal cell walls (Fig. 4.8 and 4.10B). Stomata of *in vitro* plants grown under 24-h light were immature or had bigger pores suggested to be due to poorly developed periclinal cell walls around them (Fig. 4.8). Findings of the present study on effect of light on stomata morphology confirmed previous results (**MIGUENS et al. 1993; ZACCHINI et al. 1997; MAJADA et al. 2001)**. Morphological differences of stomata

under 16-h and 24-h photoperiods could be due to differential activation of photoreceptor pigments, particularly phytochromes **(ZACCHINI et al. 1997)**. Stomatal morphology has an influence on the photosynthetic process which is critical for the growth of plants both *in vitro* and *ex vitro*. The development of photosynthetic tissues under *in vitro* conditions is a major factor determining the survival of plants *ex vitro* **(YANG AND YEH 2008)**.

TABLE 4.7 Stomata characteristics of *in vitro* plantlets growing under 16-h light and 24-h photoperiods

Treatment	[1]No. of stomata (mm^{-2})	[2]Stomata length (µm)	[2]Stomata width (µm)
16-h photoperiod	206.6 ± 15.28 b	15.8 ± 0.36 a	14.5 ± 0.26 a
24-h photoperiod	134.6 ± 12.98 a	18.4 ± 0.47 b	17.7 ± 0.37 b
[3]Control	221.9 ± 13.99 b	18.3 ± 0.39 b	14.8 ± 0.23 a

[1]Data values represent the mean ± SE (n = 4).

[2]Data values represent the mean ± SE (n = 40).

[3]Control plants were grown under a 16-h photoperiod in a growth chamber in which CO_2 was not a limiting factor. The plants were propagated conventionally from seed.

Values in the same column with different letters are significantly different ($p \leq 0.05$).

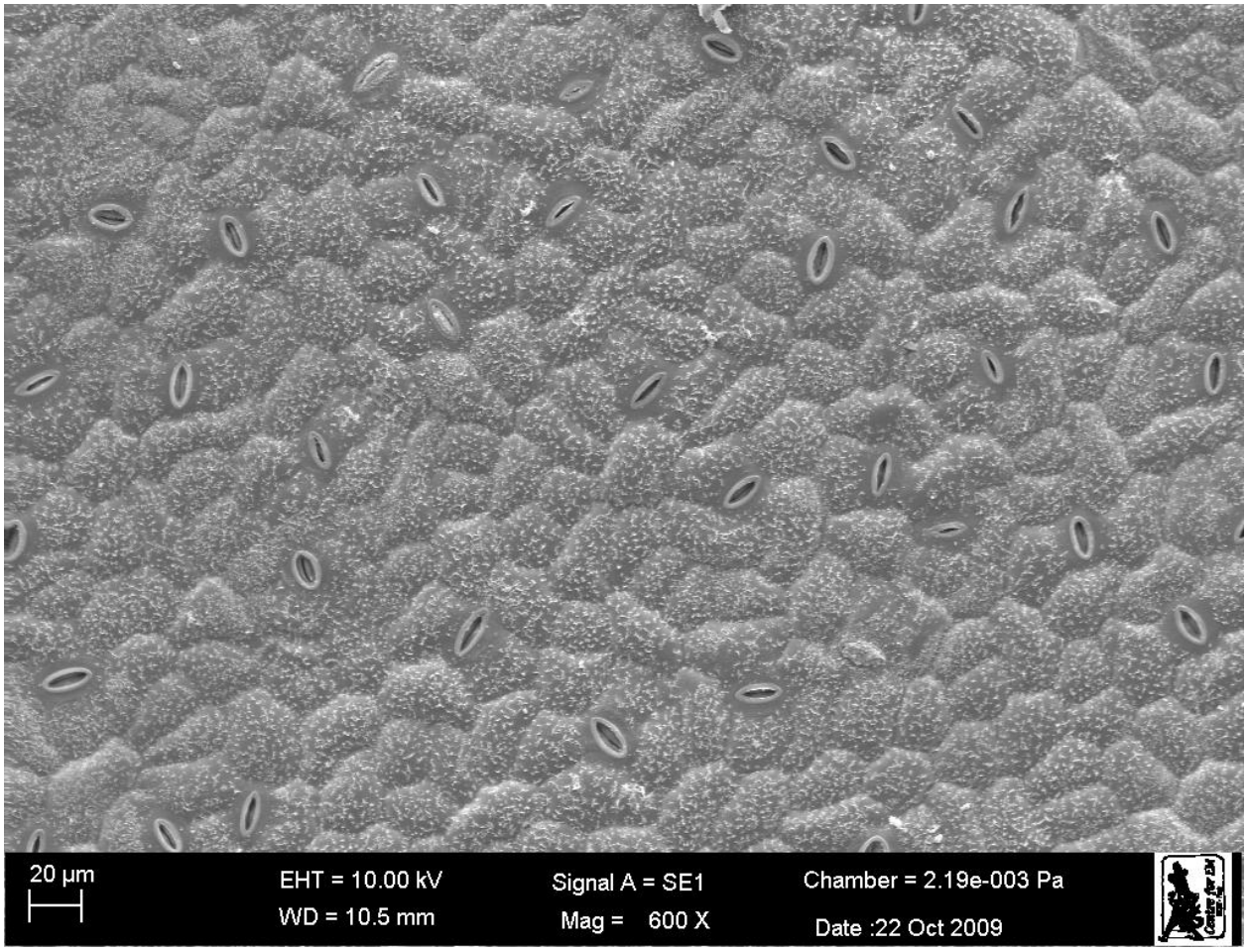


FIGURE 4.7 Structure of stomata for *in vitro* plants growing under 16-h photoperiod. Magnification = 600X.

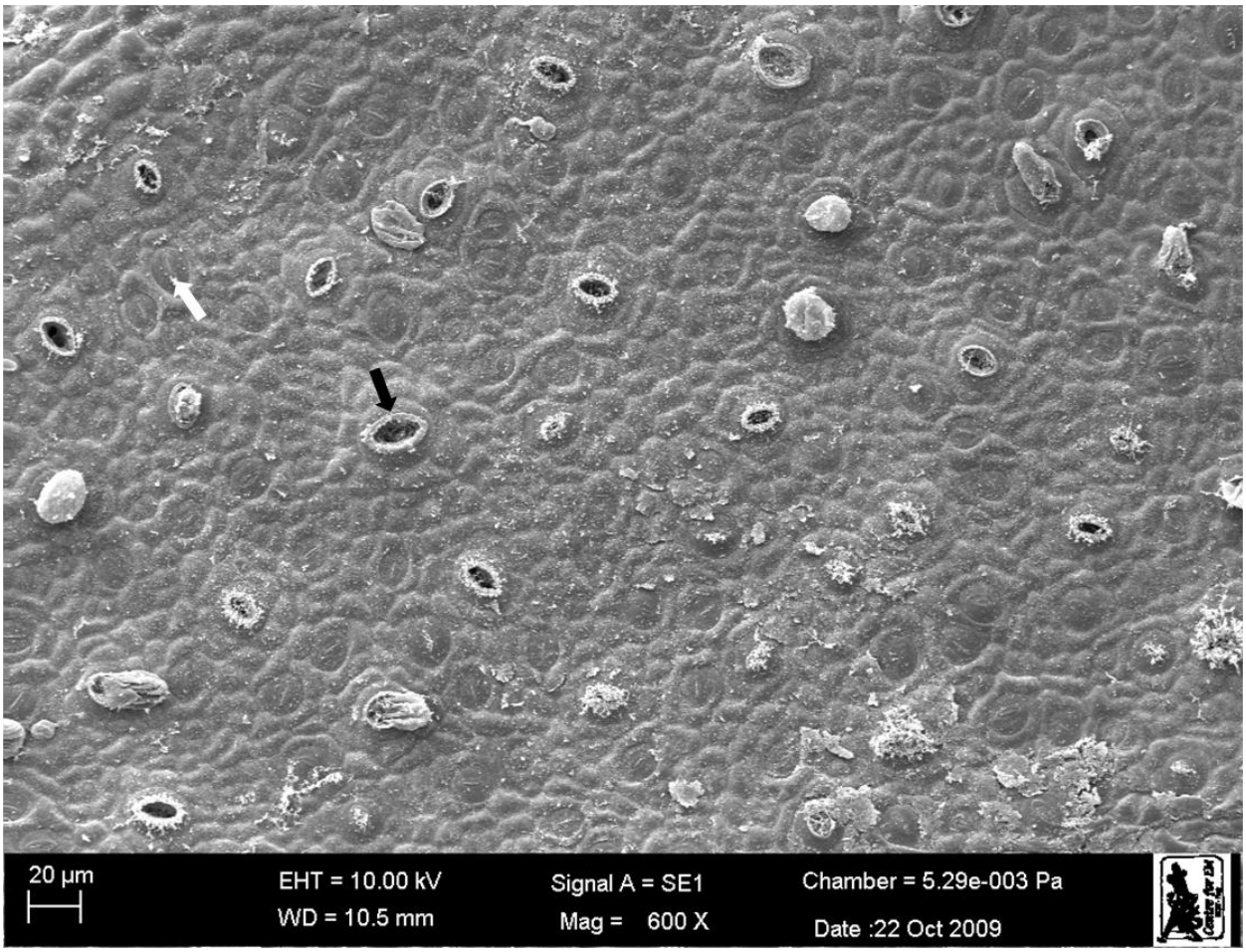


FIGURE 4.8 Structure of stomata of *in vitro* plants growing under a 24-h photoperiod showing immature (white arrow) and abnormal (solid arrow) stomata. Magnification = 600X.

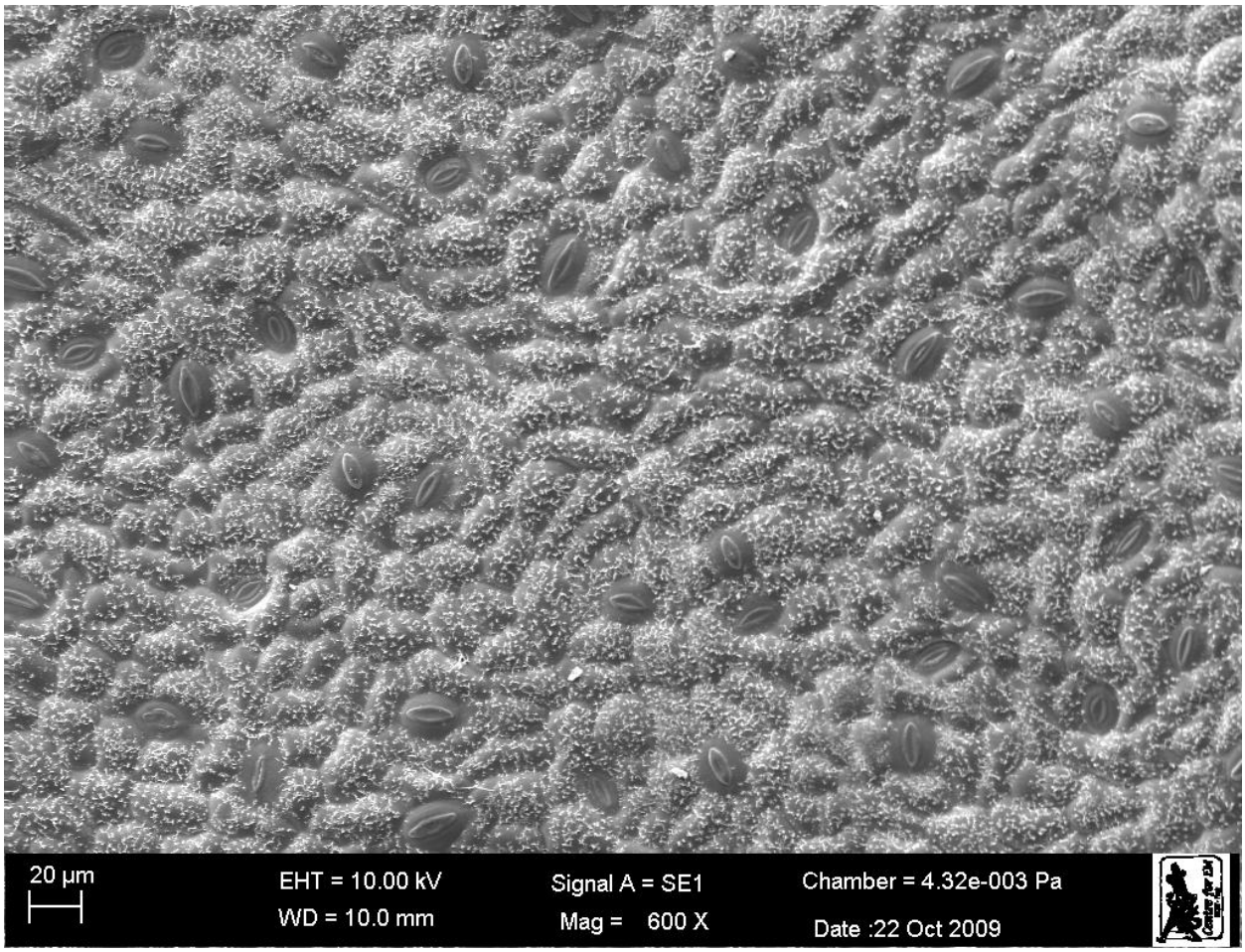


FIGURE 4.9 Structure of stomata for control plants growing under 16-h photoperiod in a growth chamber. Magnification = 600X.

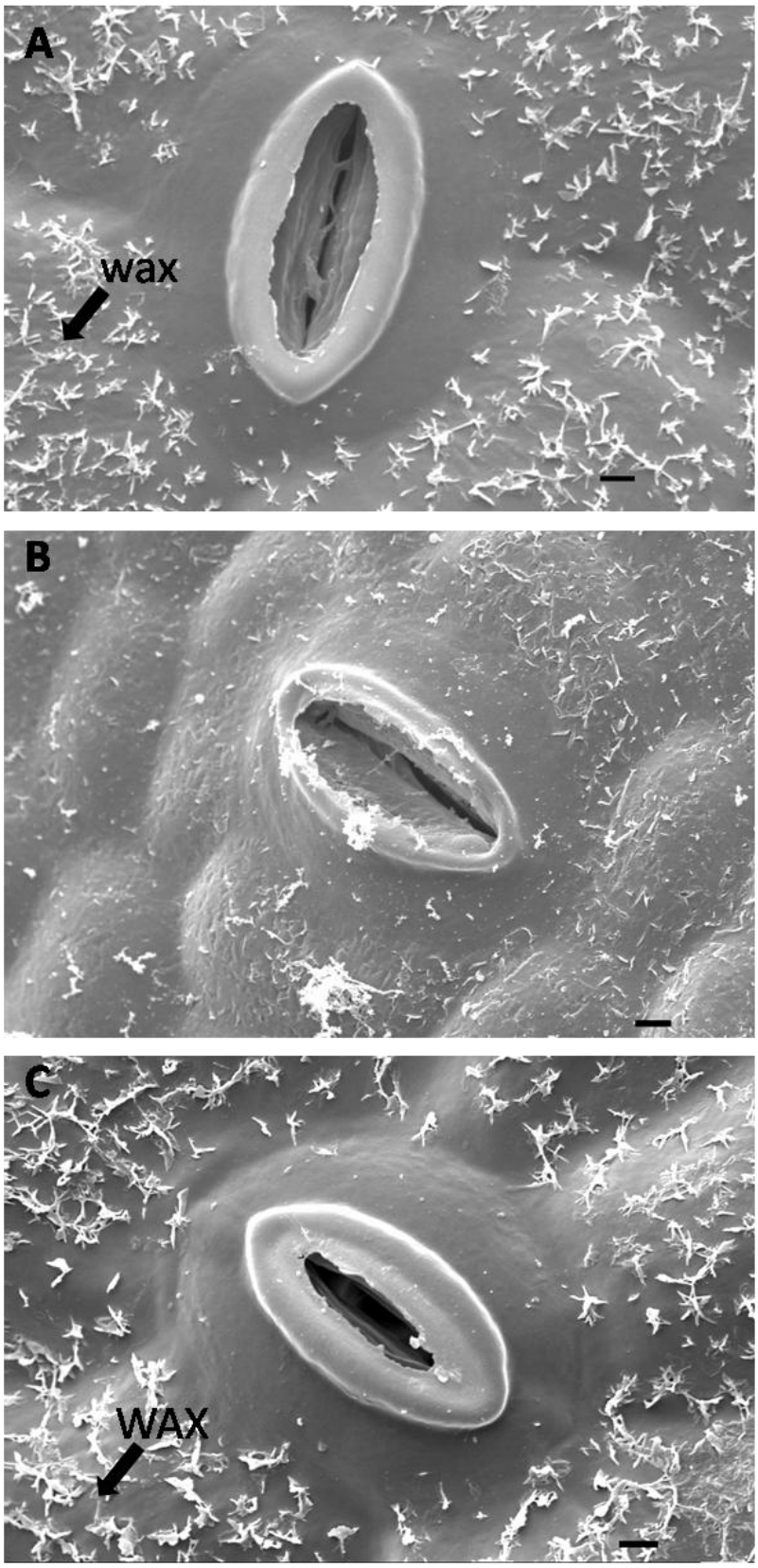


FIGURE 4.10 Structure of individual stomata under different photoperiods - **(A)** 16-h photoperiod, **(B)** 24-h photoperiod and **(C)** control plants grown in a growth chamber under a 16-h photoperiod. Magnification = 7000X. Scale bar = 2 µm.

CONCLUSIONS

Adventitious shoot and root induction was achieved using different cytokinins and auxins. Shoot organogenesis from both shoots and hypocotyls was highest on MS medium supplemented with *m*T. *Meta*-topolin also produced better results than BA, *m*TR and *mem*TR in terms of multiplication rate and size of shoots (fresh weight). Hypocotyls were more regenerative compared to epicotyls over the tested equimolar concentrations. Indole-3-butyric acid was more effective in rhizogenic induction of shoots compared to IAA and NAA. However, a challenge still remains in acclimating the *in vitro* rooted plantlets. Survival rate of plantlets *ex vitro* may be achieved by prolonging the *in vitro* period. Stomatal density was high under the 16-h photoperiod, and mature normal stomata were also observed in this treatment. The 16-h photoperiod was more ideal for micropropagation of *S. birrea* than was the 24-h photoperiod.

CHAPTER 5
PHYTOCHEMICAL AND ANTIOXIDANT EVALUATION

5.0 INTRODUCTION

In recent times, there has been a growing awareness about the possible health risks associated with synthetic food additives such as antioxidants. This realisation has stimulated an upsurge in research into naturally occurring products, particularly from medicinal plants, in search of alternative food additives. The potential and targeting of medicinal plants as possible sources of natural antioxidants for food and pharmaceutical products stems from their long-standing use in folk medicine worldwide. In China, for example, medicinal plants have been consumed for thousands of years and are reported to possess natural antioxidants with more potent activities compared to dietary vegetable and herbal plants **(WONG et al. 2005)**. **GILLES et al. (2010)** reported the approved use of *Eucalyptus* leaf extracts as food additives and cosmetic formulation ingredients in Australia, based on the utilisation of these species in traditional medicine. Natural antioxidants that occur in medicinal plants act as free-radical scavengers and chain breakers, pro-oxidant metal ion complexers, and quenchers of singlet oxygen formation **(AMAROWICZ et al. 2004)**. The antioxidant bioactivity of medicinal plants is partly attributed to phenolic compounds, which constitute a diverse and ubiquitous group of phytochemicals in the plant kingdom **(ROBARDS et al. 1999)**. Depending on their concentration, phenolic compounds have a dual bioactive role in plants, acting as both antioxidant and pro-oxidant agents at low and high concentrations, respectively **(ROBARDS et al. 1999)**. The antioxidative capacity of phenolic compounds has instigated the search for natural antioxidants as alternatives to the available synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*- butylhydroxyquinone (TBHQ) that are widely used in the food and pharmaceutical industries. The dietary intake of synthetic antioxidants, for example, BHT, could be toxic at high concentrations **(DE OLIVEIRA et al. 2009; PRASAD et al. 2009)**. In contrast, natural antioxidants are often presumed to be safe for consumption due to their plant origin **(BECKER et al. 2004)**, but this may vary depending on the plant species and environmental factors that affect growth.

Sclerocarya birrea has long been used in sub-Saharan Africa as a medicinal remedy for numerous ailments **(ELOFF 2001)**. *Sclerocarya birrea* is used in folk medicine in most parts of the sub-Saharan Africa region and it is believed to possess several therapeutic properties. Plant parts of *S. birrea,* namely the leaves, roots and bark are used to treat several conditions including diarrhoea, hypertension, diabetes, dysentery and inflammations **(BRACA et al. 2003)**. Both bark and leaf extracts of *S. birrea* have been shown to have antimicrobial effects **(ELOFF 2001)**. The purpose of the present study was to evaluate the phytochemical composition and antioxidant bioactivities of *S. birrea* young stem, leaf and opercula in aqueous methanolic extracts.

5.1 MATERIALS AND METHODS

5.1.1 CHEMICALS

Folin & Ciocalteu's phenol reagent, gallic acid (3,4,5–Trihydroxybenzoic acid), vanillin (4–hydroxyl–3 methoxybenzaldehyde), catechin, 2,2–diphenyl–1–picryl hydrazyl (DPPH), rhodanine, and *β*–carotene were obtained from Sigma–Aldrich Co. (Steinheim, Germany); sodium hydrogen carbonate, butylated hydroxytoluene (BHT) and potassium ferricyanide from BDH Chemicals Ltd (Poole, England); trichloroacetic acid, ascorbic acid, polyoxyethylene sorbitan monolaurate (Tween 20), ferric chloride ($FeCl_3$) and methanol from Merck KGaA (Darmstadt, Germany). Other chemicals used in the assays were also of analytical grade.

5.1.2 SAMPLE PREPARATION

Sclerocarya birrea plant materials were collected in November 2008 from St. Lucia, KwaZulu-Natal, South Africa. Plant parts were separated into leaves and young stems and oven-dried at 50 °C for 3 days. A voucher specimen (MOYO1) was deposited in the University of KwaZulu-Natal Herbarium (NU). Opercula were removed from marula nuts. The dried materials were ground into fine powders using a grinder (IKA®, USA). The finely ground samples (2 g) were extracted with 10 ml of 50% methanol in a sonication bath (Branson model 5210, Branson Ultrasonics B.V., Soest, Netherlands) for 20 min with temperature being kept low by adding ice to the sonication bath. The methanolic extracts were filtered under vacuum through

Whatman No. 1 filter paper and the filtrate was immediately used for the determination of total phenolics, flavonoids, gallotannins and proanthocyanidins. Dried plant extracts for antioxidant assays were ground into a fine powder, extracted with 50% methanol in a sonication bath for 20 min (1.0 g of extract per 20 ml of extraction solvent), concentrated in a rotary vacuum evaporator (Büchi, Switzerland) at a temperature of 30 °C, and transferred to sample bottles. The concentrated extracts were then dried over a stream of cold air dry weight at room temperature and stored in the dark at 10 °C. The dried methanolic extracts were re-dissolved in 50% aqueous methanol to known concentrations and immediately used for the determination of antioxidant activities.

5.1.3 QUANTIFICATION OF TOTAL PHENOLIC CONTENT

The quantification of total phenolic compounds was done according to the Folin & Ciocalteu assay as described by **MAKKAR (1999)** with modifications, using gallic acid as a standard. The reaction mixture consisted of 50 µl of the sample, 950 µl distilled water, 500 µl of 1 N Folin & Ciocalteu's phenol reagent and 2.5 ml of 2% sodium carbonate. The reaction mixtures were incubated at room temperature for 40 min. Absorbance was then measured at 725 nm using a Cary 50 UV-Visible spectrophotometer (Varian, Australia). The same spectrophotometer was used for all the assays reported hereafter unless stated otherwise. A reaction mixture that contained 50% aqueous methanol instead of sample extracts was used as a blank. The assay was done in triplicate. A standard curve of gallic acid equivalents (GAE) was used to convert the measured absorbance readings to phenolic compound concentrations per g of extract.

5.1.4 DETERMINATION OF PROANTHOCYANIDINS

Proanthocyanidins were determined using the butanol–HCl assay as described by **MAKKAR (1999)** with modifications. Three ml of butanol–HCl reagent (95:5 v/v) were added to 0.5 ml of extract followed by 0.1 ml of ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The reaction mixtures were vortexed and incubated in a boiling water bath for 1 h. After the incubation period, absorbance at 550 nm was read using a UV-Visible spectrophotometer. Absorbance was measured against a proper blank that contained the sample extract (500 µl), butanol–HCl

reagent (3 ml) and ferric reagent (100 µl), but without heating. The assay was performed in triplicate. The concentration of proanthocyanidins (%) was expressed as leucocyanidin equivalents using the formula described by **PORTER et al. (1986)** as follows:

Proanthocyanidins (%) = (A_{550} × 78.26 × dilution factor) (Equation 5.1)

where A_{550} is the absorbance at 550 nm. The dilution factor was 1.0 for all the extracts. The formula assumes the effective $E^{1\%,\ 1cm,\ 550nm}$ of leucocyanidin to be 460.

5.1.5 DETERMINATION OF GALLOTANNINS

The rhodanine assay was used to determine the concentration of gallotannins in the extracts. Each extract (50 µl) was diluted with distilled water (1.0 ml), to which 0.4 N sulphuric acid (100 µl) and rhodanine (600 µl) were added. After 5 min of incubation at room temperature, 0.5 N potassium hydroxide (200 µl) was added to the reaction mixture, followed by distilled water (4 ml) after a further 2½ min. Each reaction mixture was incubated for a further 15 min at room temperature. Absorbance at 520 nm was measured using a UV-Visible spectrophotometer. A reaction mixture containing 50% aqueous methanol instead of sample was used as a blank. The assay was done in triplicate. The concentration of gallotannins was expressed as gallic acid equivalents (GAE) per g of extract.

5.1.6 DETERMINATION OF FLAVONOIDS

Flavonoids were measured as described by **MAKKAR (1999)** with modifications. Fifty µl of each extract was diluted with distilled water to a volume of 1.0 ml. Methanol–HCl (2.5 ml, 95:5 v/v) and vanillin reagent (2.5 ml, 1.0 g 100 ml^{-1}) were added to each reaction mixture, and incubated for 20 min at room temperature. Subsequently, absorbance at 500 nm was measured using a UV-Visible spectrophotometer. A reaction mixture containing 50% aqueous methanol instead of sample extracts was used as a blank. The presence of flavonoids was indicated by a pink colouration. The assay was done in triplicate, and the concentration of flavonoids in the test samples was expressed as mg g^{-1} of extract based on a catechin equivalent (CE) standard curve.

5.1.7 DPPH (2,2–DIPHENYL–1–PICRYL HYDRAZYL) RADICAL SCAVENGING ACTIVITY

The DPPH assay for the determination of free radical scavenging activity was done as described by **KARIOTI et al. (2004)** with modifications. Fifteen µl of each plant extract was diluted with methanol (735 µl) and then added to a methanolic DPPH solution (750 µl, 0.1 mM) to give a final volume of 1.5 ml in the reaction mixture. The concentration of DPPH in the final reaction was 50 µM **(SHARMA and BHAT 2009)**, and the DPPH solution was prepared freshly before the assay. The sample extracts consisted of final concentrations of 0.65, 2.6, 5.2, 10.4, 65, 125, 250 and 500 µg ml^{-1} in the assay. The reaction mixtures were prepared under dim light and incubated at room temperature for 30 min in the dark. Decrease in the purple colouration of the reaction mixtures was read at 517 nm in a UV-Visible spectrophotometer. A standard antioxidant, ascorbic acid was used as a positive control at final concentrations of 0.88, 1.76, 3.52, 7.04 and 14.00 µg ml^{-1} in the assay. A solution containing 50% aqueous methanol instead of sample extracts or standard antioxidant was used as the negative control. Absolute methanol was used as a blank. Background correction of the sample absorbance (without DPPH) was done by subtracting the absorbance readings of the sample extracts from the corresponding readings obtained in the presence of DPPH **(KARIOTI et al. 2004)**. Each sample extract was replicated three times. The free radical scavenging activity (RSA) as determined by the decolouration of the DPPH solution was calculated according to the formula:

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D) \qquad \text{(Equation 5.2)}$$

where A_E is the absorbance of the reaction mixture containing the sample extract or standard antioxidant, and A_D is the absorbance of the DPPH solution only. Radical scavenging activity (%) was plotted against the sample extract concentration. The EC_{50} values (amount of extract required to decrease the absorbance of DPPH by 50%) were calculated from the normalised logarithmic regression curve derived from the plot data.

5.1.8 FERRIC-REDUCING ANTIOXIDANT POWER ASSAY

The ferric reducing powers of the extracts were determined based on the method of **LIM et al. (2009)** with modifications. A 30 µl volume of each plant extract, ascorbic acid or BHT (0.26 mg ml^{-1}) dissolved in methanol was added to a 96-well microplate and serially diluted. Subsequently, potassium phosphate buffer (40 µl, 0.2 M, pH 7.2) and potassium ferricyanide (40 µl, 1% w/v) were added. The reaction mixtures were incubated at 50 °C for 20 min. After the incubation period, trichloroacetic acid (40 µl, 10% w/v), distilled water (150 µl) and $FeCl_3$ (30 µl, 0.1% w/v) were added, followed by a second incubation at room temperature for 30 min in the dark. Absorbance was measured at 630 nm using a microplate reader (Opsys MR^{TM} microplate reader, Dynex Technologies Inc.). The ferric-reducing power capacities of the plant extracts and standard antioxidants were expressed graphically by plotting absorbance against concentration. Samples for the assay were prepared in triplicate.

5.1.9 β-CAROTENE-LINOLEIC ACID MODEL SYSTEM

The coupled inhibition of *β*-carotene and linoleic acid oxidation was measured according to the method described by **AMAROWICZ et al. (2004)** with modifications. *β*-Carotene (10 mg) was dissolved in 10 ml chloroform in a brown Schott bottle. Excess chloroform was evaporated under vacuum leaving a thin film of *β*-carotene. Linoleic acid (200 µl) and Tween 20 (2 ml) were then immediately added to the *β*-carotene. Aerated distilled water (497.8 ml) was added to the mixture, to give a final *β*-carotene concentration of 20 µg ml^{-1}. The mixture was further saturated with oxygen by vigorous agitation to form an orange coloured emulsion. The emulsion (4.8 ml) was dispensed into test tubes to which sample extracts or BHT (200 µl, 6.25 mg ml^{-1}) were added, giving a final concentration of 250 µg ml^{-1} in the assay. Initial absorbance at 470 nm (t = 0) for each reaction mixture was measured immediately after adding the sample extract or BHT. Subsequent absorbance values were obtained every 30 min for 2 h, with incubation in a water bath at 50 °C. A Tween 20 solution was used as a blank. The negative control consisted of 50% aqueous methanol being added in place of the sample extract. The rate of *β*-carotene bleaching was calculated using the following formula:

Rate of *β*-carotene bleaching = $\ln (A_{t=0}/A_{t=t}) \times 1/t$ (Equation 5.3)

where $A_{t=0}$ is the absorbance of the emulsion at 0 min; and $A_{t=t}$ is the absorbance at time *t* (30, 60, 90 min). The average rate of *β*-carotene bleaching was then calculated based on rates at 30, 60 and 90 min. The calculated average rates were used to determine the antioxidant activity (ANT) of the sample extracts, and expressed as percentage inhibition of the rate of *β*-carotene bleaching using the formula:

$$\% \text{ ANT} = (R_{control} - R_{sample})/ R_{control} \times 100 \quad \text{(Equation 5.4)}$$

where $R_{control}$ and R_{sample} represent the respective average *β*-carotene bleaching rates for the negative control and plant extracts. Antioxidant activity was further expressed as the oxidation rate ratio (ORR) based on the equation:

$$\text{ORR} = R_{sample}/ R_{control} \quad \text{(Equation 5.5)}$$

Antioxidant activity (AA) was calculated as described by **BRACA et al. (2003)** based on the inhibition of coupled oxidation of *β*-carotene and linoleic acid against the negative control at *t* = 60 min and *t* = 120 min using the formula:

$$\%\text{AA} = [1 - (A_0 - A_t)]/(A_{00} - A_{0t}) \times 100 \quad \text{(Equation 5.6)}$$

where A_0 is the absorbance of the sample extract at the beginning of incubation; A_t is the absorbance at time *t* = 60 and 120 min for the sample extract; and A_{00} and A_{0t} represent the absorbance of the negative control (without sample extract) at the beginning of incubation and at time *t* = 60 and 120 min, respectively.

5.1.10 STATISTICAL ANALYSIS

All determinations were done in triplicate, and the results reported as means ± standard error of the mean (SE). One-way analysis of variance (ANOVA) was done for the comparison of mean values, which were further separated using Duncan Multiple Range Test ($P \leq 0.05$). Statistical analyses were done using SPSS version 15.0 for Windows (SPSS Inc., USA). Regression analyses, and graphs were constructed using either GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., USA) or SigmaPlot 2002 for Windows version 8.0 (SPSS Inc., USA). Calculation of EC_{50} values was done using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., USA).

5.2 RESULTS AND DISCUSSION

5.2.1 PHYTOCHEMICAL CONSTITUENTS

The total phenolic content of *S. birrea* was relatively high for all plant part extracts tested (Table 5.1). Extracts of young stems of *S. birrea* contained the highest total phenolic content of 14.15 ± 0.03 mg GAE g^{-1} of extract. Compared to young stems, leaf extracts of *S. birrea* had a comparatively lower total phenolic content of 13.95 ± 0.05 mg GAE g^{-1} of extract. The lowest phenolic content (2.46 ± 0.03 mg GAE g^{-1} of extract) was recorded for *S. birrea* opercula extracts. Although phenolic compounds are found in virtually all plants and plant parts, their quantitative distribution varies between and within plant species, and between different organs in a plant **(ROBARDS et al. 1999)**. Plants with high levels of phenolic compounds have been shown to exhibit high antioxidant capacities **(KATSUBE et al. 2004)**. Methanolic extracts of *Anarcadium occidentale* shoots had high levels of phenolic compounds and corresponding potent antioxidant activities **(RAZALI et al. 2008)**.

Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers **(HAKKIM et al. 2007)**. The high content of phenolic compounds observed for young stem and leaf extracts suggest that these plant parts have high antioxidant potential. Phenolic compounds are also known to have antibacterial, antifungal and anti-inflammatory properties **(ARAÚJO et al. 2008)**.

The highest concentration of proanthocyanidins was recorded for *S. birrea* leaves (1.25%), which was 9-fold the level in *S. birrea* opercula extracts (Table 5.1). *Sclerocarya birrea* young stem extracts also had comparatively high concentrations of proanthocyanidins, of 1.16%. Opercula extracts had the lowest proanthocyanidins content of 0.14%. The anti-inflammatory, antifungal, antioxidant and healing properties of some plant extracts have been attributed to the presence of tannins **(ARAÚJO et al. 2008)**. Tannins can also reduce metallic ions such as Fe^{3+} to the Fe^{2+} form, and can inhibit the 5-lipoxygenase enzyme in arachidonic acid metabolism, which is important in inflammation physiology **(OKUDA 2005)**.

Similar to the trend for total phenolic content, the concentration of hydrolysable tannins as measured by the level of gallotannins was highest for *S. birrea* young stem at 246.12 ± 3.76 µg GAE g^{-1} of extract (Table 5.1). The level of gallotannins was comparatively 6-7 times lower for *S. birrea* opercula extracts (31.83 ± 2.01 µg GAE g^{-1} of extract). The concentration of gallotannins in leaf extracts was moderate (88.48 ± 4.42 µg GAE g^{-1}) compared to young stem and opercula extracts.

The flavonoid content of the different extracts ranged from 80.93 ± 5.53 to 1219.39 ± 16.62 µg CE g^{-1} of extract (Table 5.1). *Sclerocarya birrea* young stem extracts contained the highest flavonoid levels (1219.39 ± 16.62 µg CE g^{-1}). The concentration of flavonoids for *S. birrea* young stem extracts was 2.5 times the level in *S. birrea* leaf extracts; and 15 times higher than *S. birrea* opercula extracts. Flavonoids constitute an important group of secondary metabolites in plants. Apart from their functions in plant physiology and biochemistry, they have been reported to exhibit high antioxidant activity, thereby offering protection against the damaging effects caused by the overproduction of reactive oxygen species **(BRACA et al. 2003)**. Flavonoids also act on enzymes and pathways involved in inflammatory processes, are strong inhibitors of lipid peroxidation, and possess antibacterial and antifungal properties **(ARAÚJO et al. 2008)**. The observations made in this study suggest that *S. birrea* extracts, in particular young stems and leaves, may be good sources of flavonoids. The consumption of *S. birrea* as medicinal remedies against inflammatory conditions, fever, pain and microbial conditions is strongly justified by the presence of these flavonoids.

TABLE 5.1 Total phenolic content, flavonoids, gallotannins and proanthocyanidins in methanolic extracts of *S. birrea* young stems, leaves and opercula

Plant part	Total phenolics (mg GAE g^{-1})	Flavonoids (µg CE g^{-1})	Gallotannins (µg GAE g^{-1})	Proanthocyanidins (%)
Leaves	13.95 ± 0.05 b	474.48 ± 9.54 b	88.48 ± 4.42 b	1.25 ± 0.002 c
Young stems	14.15 ± 0.03 c	1219.39 ± 16.62 c	246.12 ± 3.76 c	1.16 ± 0.005 b
Opercula	2.46 ± 0.03 a	80.93 ± 5.53 a	31.83 ± 2.01a	0.14 ± 0.006 a

Data represent mean ± SE of three separate measurements.

Different letters in the same column represent significantly different values ($p \leq 0.05$) as separated by Duncan Multiple Range Test.

5.2.2 DPPH (2,2–DIPHENYL–1–PICRYL HYDRAZYL) RADICAL SCAVENGING ACTIVITY

Fig. 5.1 depicts the dose-dependent DPPH radical scavenging activity of *S. birrea* extracts expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH solution without plant extracts. All the plant extracts showed a propensity to quench DPPH free radicals as indicated by the exponential increase in radical scavenging activity (%). This corresponded to a rapid decrease in absorbance in the presence of a plant extract, indicating high antioxidant potency of the extracts in terms of electron or hydrogen atom-donating capacity **(AMAROWICZ et al. 2004)**. The EC_{50} values of the extracts ranged from 5.028 to 6.921 µg ml^{-1} (Table 5.2). *Sclerocarya birrea* young stem extracts exhibited the highest DPPH radical scavenging activity with an EC_{50} value of 5.028 µg ml^{-1}. The EC_{50} value for *S. birrea* leaves was relatively high (5.602 µg ml^{-1}). Both young stems and leaves were more potent compared to ascorbic acid (6.868 µg ml^{-1}), which is a standard antioxidant. Young stem extracts also had the highest concentration of total phenolic content, flavonoids and gallotannins. Similarly, **KATSUBE et al. (2004)** reported a high correlation coefficient (*R = 0.969*) between the Folin & Ciocalteu assay (total phenolic content) and the DPPH radical scavenging assay of some Japanese medicinal plants.

TABLE 5.2 DPPH radical scavenging activity of *S. birrea* methanolic extracts

Plant part	EC_{50} (µg ml^{-1})	R^2
Leaves	5.602	0.9643
Young stems	5.028	0.9735
Opercula	6.921	0.9706
Control (Ascorbic acid)	6.868	0.9853

Data represent means of three replicates.

R^2 which is the coefficient of determination measures the goodness of fit of the curves.

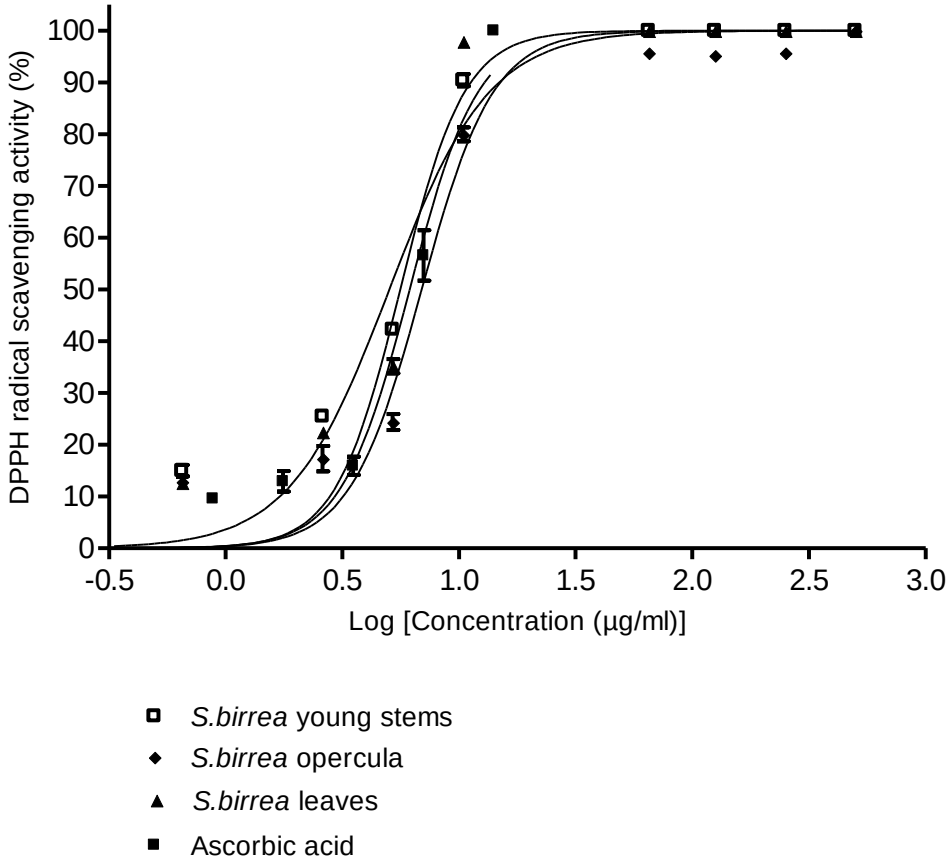


FIGURE 5.1 Dose-dependent DPPH free radical scavenging activity (%) of *S. birrea* young stems, leaves and opercula methanolic extracts. Bars = SE.

5.2.3 FERRIC-REDUCING ANTIOXIDANT POWER ASSAY

Antioxidant activities of *S. birrea* young stem, leaf and opercula extracts were assessed for their ability to reduce the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. The ferrous ion was monitored by measuring the formation of Perl's Prussian blue at 630 nm using an Opsys MR^{TM} microplate reader **(LAI et al. 2001)**. Fig. 5.2 presents the dose-dependent ferric-reducing powers of the sample extracts, ascorbic acid and BHT. The reducing power of all the sample extracts (young stems, leaves and opercula), ascorbic acid and BHT increased with increasing concentration. The reducing power of ascorbic acid was significantly more pronounced relative to the plant extracts. However, the antioxidant potencies of BHT, young stem and leaf extracts were comparable. *Sclerocarya birrea* opercula extracts exhibited the lowest activity. Nevertheless, *S. birrea* young stem, leaf and opercula extracts have the

capacity to act as electron donors, indicating their potential to react with free radicals, which they can convert to more stable products.

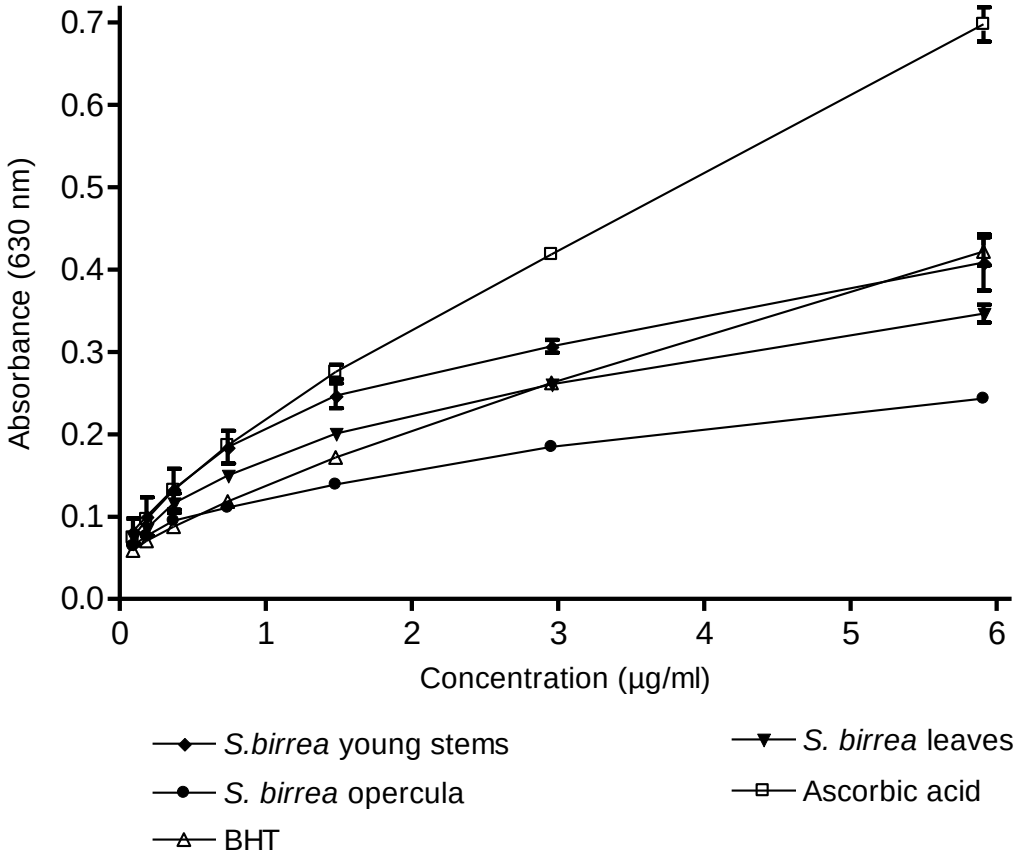


FIGURE 5.2 Ferric ion-reducing power effects of *S. birrea* methanolic plant extracts showing a dose-dependent linear increase with absorbance. Bars = SE.

The phenolic content of *S. birrea* young stems was 6-fold higher than that of opercula extracts (Table 5.1), and a similar trend was observed in the ferric-reducing antioxidant power assay. For example, an absorbance reading of 0.2 corresponded to a dose level of approximately 0.8 and 5.0 µg ml^{-1} for *S. birrea* young stems, and opercula extracts, respectively. Thus, to achieve a similar extent of reducing power as *S. birrea* young stem extracts, a 6-fold dose level is required for *S. birrea* opercula extracts. **SIDDHURAJU et al. (2002)** as well as **ANWAR et al. (2007)** also reported a direct relationship between reducing power effects of plant extracts to the content of phenolic compounds. Due to the high total phenolic content of sample extracts, it

is postulated that they represent the primary source of this antioxidant activity **(JONFIA-ESSIEN et al. 2008)**.

5.2.4 *β*-CAROTENE-LINOLEIC ACID COUPLED OXIDATION

This assay measures the ability of a plant extract to prevent or minimise the coupled oxidation of *β*-carotene and linoleic acid in an emulsified aqueous system **(PAREJO et al. 2002)**. In the reaction, the emulsion loses its orange colour due to the reaction with radicals, but this process can be inhibited by antioxidants. Oxidation reactions result in the bleaching of carotenoids, and the process is induced by light, heat or peroxyl radicals **(HASSAS-ROUDSARI et al. 2009)**. Heat-induced coupled oxidation of an aqueous emulsion system of *β*-carotene and linoleic acid was used to assess the antioxidant activity of *S. birrea* young stem, leaf and opercula extracts. Table 5.3 presents the antioxidant activities of the extracts. The antioxidant activity calculated based on the average rate of *β*-carotene bleaching was relatively high for all the plant extracts (89.6 to 93.9%). The lowest potency (89.6%) in the assay was recorded for *S. birrea* young stem extracts. The oxidation rate ratio (ORR) ranged from 0.0603 to 0.103 for all the tested extracts. The lowest ORR value corresponds to extracts with the highest antioxidant capacity, hence *S. birrea* leaf extracts had the highest antioxidant activity. The oxidation rate ratios of the extracts were all significantly higher that of the control (BHT). Antioxidant activity (AA) was determined on the basis of inhibition of coupled oxidation of *β*-carotene and linoleic acid at t = 60 min and t = 120 min. The antioxidant activity ranged from 46.7 to 70.3% (t = 60 min), and 44.1 to 53.5% (t = 120 min).

For all the indicators used to measure antioxidant activities of *S. birrea* plant extracts, young stems exhibited the lowest activity in the assay. However, for all the tested indicators leaf extracts showed superior antioxidant activity compared to the control (BHT). Figure 5.3 presents the absorbance of sample extracts and BHT plotted against time over 2 h. In the absence of potent antioxidants the orange colour of *β*-carotene is bleached resulting in a relatively rapid reduction in absorbance at 470 nm. The presence of phenolic antioxidants prevents or reduces the bleaching of *β*-carotene by quenching peroxyl radicals **(ANWAR et al. 2007)**. Lipids such as linoleic acid form a peroxyl radical in the presence of reactive oxygen species (ROS)

and oxygen. The peroxyl radical reacts with *β*-carotene to form a stable *β*-carotene radical, which subsequently leads to the bleaching of its orange colour **(MOON and SHIBAMOTO 2009)**. In the presence of an antioxidant, it reacts competitively with the peroxyl radical, thereby preventing or minimising the bleaching of *β*-carotene **(MOON and SHIBAMOTO 2009)**. The plant extracts were able to quench the peroxyl radicals thereby reducing the coupled oxidation of *β*-carotene and linoleic acid. The beneficial effects of antioxidants as therapeutic agents are well documented in the literature. The use of *S. birrea* in traditional medicine may provide protection against oxidative stress caused by harmful free radicals at the cellular level. These plant extracts are also potential sources for the development of natural antioxidants for the food and pharmaceutical industries.

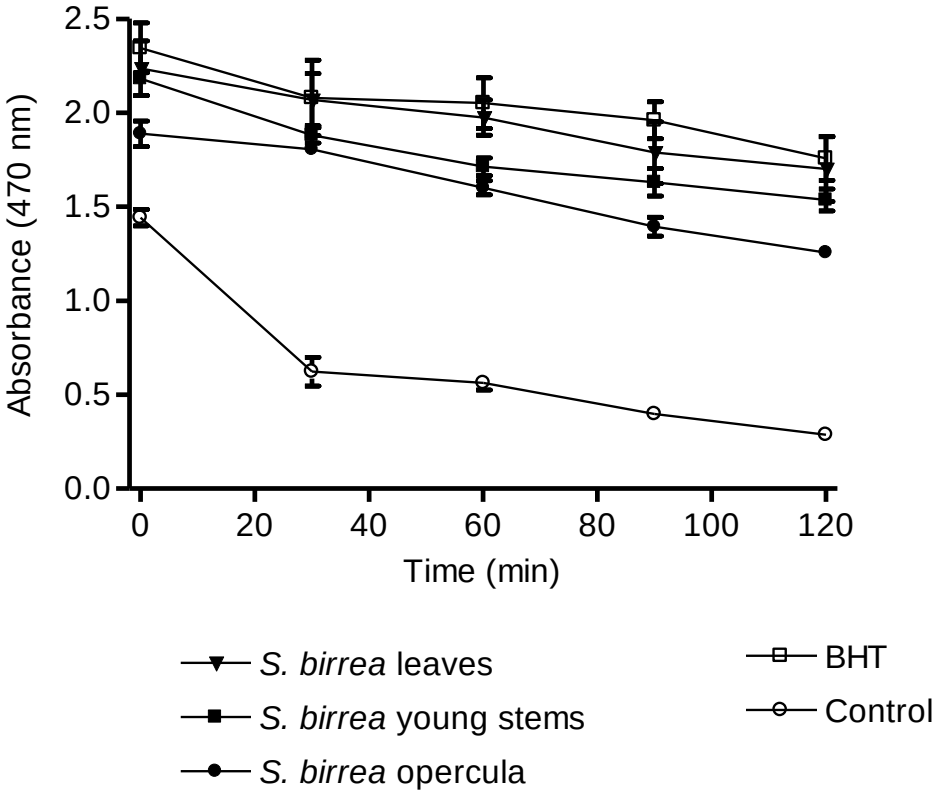


FIGURE 5.3 Antioxidant activity of *S. birrea* methanolic extracts as evaluated in the *β*-carotene-linoleic acid coupled oxidation model system. Bars = SE.

TABLE 5.3 Antioxidant activity of *S. birrea* methanolic extracts as assessed by the coupled oxidation of *β*-carotene and linoleic acid

Plant part	ANT (%)[a]	ORR [b]	AA_{60} [c]	AA_{120} [d]
Leaves	93.9 ± 1.16 b	0.0603 ± 0.011 a	70.3 ± 11.1 b	53.5 ± 8.50 b
Young stems	89.6 ± 1.76 ab	0.103 ± 0.017 ab	46.7 ± 7.97 ab	44.1 ± 6.84 ab
Opercula	93.6 ± 2.21 b	0.063 ± 0.022 a	67.3 ± 10.6 b	45.2 ± 7.78 b
Control (BHT)	92.8 ± 2.78 b	0.158 ± 0.019 b	66.5 ± 9.13 b	49.0 ± 5.71 b

[a] ANT (%) - Antioxidant activity calculated on the basis of the rate of *β*-carotene bleaching at *t* = 30, 60 and 90 min.

[b] ORR - Oxidation rate ratio. The lower the ratio the more active the extract.

[c] AA_{60} - % Antioxidant activity of a plant extract or control (BHT) at *t* = 60 min.

[d] AA_{120} - % Antioxidant activity of an extract or control (BHT) at *t* = 120 min.

5.3 CONCLUSIONS

Excessive production of free radicals has been shown to be involved in the pathogenesis of a number of diseases **(BRACA et al. 2003)**. On the other hand, there is widespread evidence that intake of natural antioxidants can avert the associated health effects caused by oxidative damage to DNA, lipids and proteins **(BECKER et al. 2004)**. *Sclerocarya birrea* plant extracts in the present study contained appreciably high levels of total phenolic compounds, proanthocyanidins, gallotannins and flavonoids. The highest concentrations of these phytochemicals were detected in *S. birrea* young stem as well as leaf extracts. All *S. birrea* sample extracts also exhibited high antioxidant and free radical scavenging activities, and some even showed higher potency than the standard synthetic antioxidants in some instances, for example leaf and young stem extracts had higher activity in the DPPH assay. The results of the present study suggest that plant extracts of *S. birrea*, in particular young stems and leaves, provide a substantial source of secondary metabolites which act as natural antioxidants and may contribute beneficially to the health of consumers. *Sclerocarya birrea* young stems and leaves may be good bio-sources for the development of antioxidant food additives.

CHAPTER 6
ANTIMICROBIAL, ANTI-INFLAMMATORY AND ACETYLCHOLINESTERASE INHIBITORY EVALUATION

6.0 INTRODUCTION

In most parts of Africa, including South Africa, the utilisation of plants for medicinal purposes is a well established practice. The growing demand for medicinal plants, possibly arising from the increasing levels of poverty and rising costs of modern medicine, continues to exert sustained pressure on the limited plant resource base, posing a major threat to biodiversity conservation. A major concern emanating from this growing demand for medicinal plants is the decimation of tree species, which have long regeneration cycles, through the harvesting of roots and mature bark. The over-exploitation of medicinal plants from their natural habitats has long been a global challenge **(JÄGER and VAN STADEN 2005)**. **MANDER (1998)** recorded tha in KwaZulu-Natal, South Africa, bark from the main tree trunk and roots from mature trees accounted for a combined total of 54% (27% each) of the medicinal market products sold by traders. Other studies in South Africa have also reported high utilisation of trees (32%) for medicinal purposes and a corresponding high preference (83%) for mature bark by traders **(ZSCHOCKE et al. 2000b)**. A number of conservation options have been proposed to curtail the prevalent destructive utilisation of medicinal plants, for example, establishment of conservation areas, law enforcement against bark collections, large-scale cultivation of popular species **(ZSCHOCKE et al. 2000b)**, plant and plant part substitution **(JÄGER and VAN STADEN 2005)**. However, most of these conservation strategies are long-term and have to be implemented inclusively with short-term options to cater for a sustained supply of medicinal remedies. Plant substitution, though practical, may be resisted by consumers who may not readily accept alternative plants **(ZSCHOCKE et al. 2000b)**. Instead, a practical alternative strategy could be plant part substitution through the utilisation of renewable parts, such as young stems and leaves which could lead to non-destructive harvesting **(JÄGER and VAN STADEN 2005)**. Thus, as part of an overall conservation strategy, medicinal plant research should include investigations into parts that are traditionally less used such as stems and leaves **(ZSCHOCKE et al. 2000b)**.

In the tropics and subtropics, Anacardiaceae species are commonly used as ethnomedicinal bio-resources. For example, *Spondias mombin* has been reported to be an important component of traditional health care in the West Indies, southern Mexico, Peru, Brazil, Equatorial Guinea, Cote D'Ivoire, Nigeria and Sierra Leone **(AYOKA et al. 2006)**. **LIMA et al. (2006)** reported on the medicinal uses of the bark, leaves, roots and flowers of *Mangifera indica* in the treatment of diarrhoea, fever, gastritis and ulcers by tribes in the tropical and subtropical regions. In South Africa, two Anacardiaceae species, *S. birrea* and *Harpephyllum caffrum*, are used extensively in folk medicine for a wide range of ailments, including diarrhoea, dysentery, malaria, fevers, headaches and inflammations. Mature bark of *S. birrea* and *H. caffrum* are the preferred plant part sold by medicinal traders for both species in KwaZulu-Natal, accounting for 7% and 5%, respectively **(MANDER 1998)**. Though the two species are not extinct, continued unchecked harvesting of the bark for medicinal purposes poses a major threat to their conservation. The purpose of this study was to evaluate renewable plant parts (young stems, leaves and opercula) of *S. birrea* for antimicrobial bioactivity, inhibition of cyclooxygenase-1 and 2 enzymes, which are involved in the biosynthesis of prostaglandins in inflammation processes and acetylcholinesterase inhibition.

6.1 MATERIALS AND METHODS

6.1.1 CHEMICALS

Yeast malt (YM) broth, Mueller-Hinton (MH) broth, neomycin, amphoterecin B, adrenaline, glutathione (reduced form), prostaglandin E_2, indomethacin, cyclooxygenase-1 (COX-1, isolated from ram seminal vesicles), cyclooxygenase-2 (COX-2, human recombinant expressed in S*f* 21 cells), arachidonic acid, [^{14}C] arachidonic acid, *p*-iodonitrotetrazolium chloride (INT), acetylthiocholine iodide (ATCI), 5,5'-dithiobio-(2-nitrobenzoic acid) (DTNB), galanthamine, acetylcholinesterase (AchE, from electric eel, type VI-S) were purchased from Sigma-Aldrich (Sigma Chemical Co., Steinheim, Germany). Silica gel 60 (particle size 0.063-0.200, 70-230 mesh ASTM), petroleum ether (PE), ethanol (EtOH), methanol, dichloromethane (DCM) and dimethyl sulphoxide (DMSO) were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals used in the assays were of analytical grade.

6.1.2 PLANT MATERIALS

Sclerocarya birrea plant materials were obtained from St. Lucia, KwaZulu-Natal, South Africa in November 2008. Plant parts were separated into leaves and young stems and oven dried at 50 °C for three days. Opercula extracts were obtained from marula nuts.

6.1.3 PREPARATION OF PLANT SAMPLES

The dried samples were ground into fine powders using a blender (IKA® All basic, Wilmington, USA). Plant samples for antimicrobial and anti-inflammatory assays were extracted sequentially with petroleum ether (PE), dichloromethane (DCM) and 80% ethanol (EtOH). Samples for the acetylcholinesterase assay were extracted non-sequentially with petroleum ether, dichloromethane and 50% methanol. Extractions were done on ice in a sonication bath (Branson model 5210, Branson Ultrasonics B.V., Soest, The Netherlands) using 20 ml of extraction solvent per gram of extract for 1 h. The extracts were vacuum-filtered through Whatman No. 1 filter paper, concentrated under vacuum (Rotavapor–R, Büchi, Switzerland) at 30 °C and dried at room temperature until a constant dry weight was obtained. The dried extracts were stored in sample bottles at 10 °C in the dark.

6.1.4 ANTIBACTERIAL ASSAY

The minimum inhibition concentration (MIC) of the plant extracts was determined using the serial micro-dilution assay **(ELOFF 1998)**. Four bacterial strains consisting of two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) strains were incubated in suspension overnight at 37 °C in a water bath on an orbital shaker. The concentration of each bacterial culture used in the assay had an absorbance value of between 0.4-0.6 at 600 nm determined with a UV-Visible spectrophotometer (Varian, Australia). The bacteria were diluted in a ratio of 1:100 with sterile MH broth, and all sample extracts were re-suspended in 80% ethanol to a concentration of 50 mg ml^{-1}. The re-suspended sample extracts (100 µl) were serially diluted with sterile distilled water (100 µl) in 96-well microtitre plates and inoculated with 100 µl of the respective bacterial strains. The microtitre plates were covered with parafilm and incubated at 37 °C for 24 h. The indicator, INT

(40 µl, 0.2 mg ml^{-1}) which was dissolved in distilled water served as the indicator of bacterial growth after incubation at 37 °C for 1 h. Wells with active bacterial growth turned reddish-pink in the presence of biologically active micro-organisms, which reduce the colourless tetrazolium salt. The MIC values corresponded to the lowest concentration of the sample extract that completely inhibited bacterial growth, i.e. the last clear well in the dilution series. A positive control, neomycin (100 µl, 100 µg ml^{-1} in the first well) was used against each bacterial strain. Ethanol, the solvent used to re-suspend the extracts, bacteria-free MH broth, sterile distilled water, and each bacterial strain, with no extract added completed the set of controls. The minimum bacterial concentration (MBC) was determined by adding MH broth (50 µl) to each of the clear wells (no bacterial growth), followed by further incubation at 37 °C for 24 h. The lowest concentration in the dilution series without bacterial growth was taken as the MBC **(KUETE et al. 2008; PAVITHRA et al. 2009)**. The assay was repeated twice with a total of four replicates.

6.1.5 ANTIFUNGAL ASSAY

The micro-dilution assay **(ELOFF 1998)** with modifications was used in the determination of antifungal activity against a standard strain of *Candida albicans* (ATCC 1023). An overnight culture of *C*. *albicans* in Yeast Malt (YM) broth was prepared and incubated at 37 °C in a water bath on an orbital shaker. Four millilitres of sterile saline (0.85% NaCl) were added to 400 µl of *C*. *albicans* culture after the overnight incubation period. The absorbance of the fungal culture was read at 530 nm and adjusted with sterile saline against a McFarland standard solution (0.5 M) to give a reading of 0.25–0.28. The prepared fungal culture was then diluted with sterile YM broth at a ratio of 1:1000 which was used in the assay. All plant extracts were re-suspended in 80% EtOH to a concentration of 50 mg ml^{-1}. One hundred µl of sterile distilled water were added to each of the 96 wells in a micro-plate and serially diluted with an equal volume of the respective plant extracts. Subsequently, 100 µl of the fungal culture were added to each micro-plate well. The standard, Amphoterecin B was prepared by adding 0.5 mg in 200 µl DMSO and this solution was further diluted 10 times with sterile distilled water to give a concentration of 0.25 mg ml^{-1}. One hundred µl of Amphoterecin B (0.25 mg ml^{-1}) was serially diluted with the test fungus served as a positive control, while 80% EtOH was used as a negative control. Other

controls were YM broth, the fungal strain with no extract, DMSO and sterile distilled water. All the microtitre plates were covered with parafilm and incubated at 37 °C for 48 h, after which INT (50 µl, 0.2 mg ml^{-1}) was added to each well. For each sample extract the MIC was indicated by the last clear well in the dilution series. The minimum fungicidal concentration (MFC) was then determined by adding YM broth (50 µl) to each of the clear wells, followed by a further incubation at 37 °C for 24 h. The MFC was also indicated by the last clear well in the dilution series. The assay was repeated twice with a total of four replicates.

6.1.6 ANTI-INFLAMMATORY ACTIVITY USING CYCLOOXYGENASE-1 AND -2 ASSAYS

Anti-inflammatory activities of the sample extracts were evaluated on their ability to inhibit prostaglandin synthesis using COX-1 and COX-2 enzyme assays as described by **ELDEEN and VAN STADEN (2008)** and **LUNDGAARD et al. (2008)**. Briefly, both cyclooxygenase assays followed the same procedure except that in the preparation of the co-factor solution double the quantity of adrenaline (0.6 mg) was used for COX-2 compared to COX-1. Tris buffer (200 µl, pH 8.0) and co-factor solution (1250 µl) were added to COX-1 or COX-2 enzyme (50 µl, 75 U) and incubated on ice for 5 min. The enzyme/co-factor solution (60 µl) was then added to sample extracts and incubated at room temperature for 5 min. The substrate, [^{14}C] arachidonic acid (20 µl, 1% v/v), was added to the sample extracts before incubation in a water bath at 37 °C for 10 min. After incubation the reaction mixtures were immediately placed on ice and enzyme reaction was terminated by adding 2 N HCl (10 µl) to sample extracts. Unlabelled prostaglandin carrier solution (4 µl, 0.2 mg ml^{-1}) was added to each sample extract mixture. Separation of [^{14}C] prostaglandins synthesised in the assay, from unmetabolised arachidonic acid was done using silica gel column chromatography. The arachidonic acid was eluted by the addition of Eluent 1 (*n*-hexane: 1.4-dioxane: glacial acetic acid, 4 ml, 350:150:1 v/v). Prostaglandins were then eluted into scintillation vials by adding Eluent 2 (acetyl acetate: methanol, 3 ml, 425:75 v/v) to each silica gel column. Following the addition of scintillation fluid (4 ml) to each vial, radioactivity was counted using a Beckman Coulter™ LS Multipurpose scintillation counter (USA). Controls for both COX-1 and COX-2 assays included a solvent blank, background and an indomethacin standard.

The solvent blank consisted of ethanol (2.5 µl) instead of sample extracts. For the background control (on ice throughout the assay), the enzyme was inactivated using 2 N HCl (10 µl) before adding ^{14}C-labelled arachidonic acid. Indomethacin (positive control) was used at a concentration of 5 µM (COX-1 assay) and 200 µM (COX-2 assay). Both COX-1 and COX-2 assays were done in duplicate and repeated twice. Inhibition of prostaglandin synthesis was calculated by comparing the radioactivity of each sample extract to that of the solvent blank using the formula:

$$\text{Inhibition (\%)} = 1-\left(\frac{DMP\ (sample\ )-DMP\ (background\ )}{DMP\ (solvent\ \ blank\ -DMP\ (background\ )}\right)\times 100 \qquad \text{(Equation 6.1)}$$

where *DMP(sample)*, *DMP(background)* and *DMP(solvent blank)* represent the radioactivity counts of the sample extract, background and solvent blank, respectively.

6.1.7 MICROTITRE-PLATE ASSAY FOR INHIBITION OF ACETYLCHOLINESTERASE

Inhibition of acetylcholinesterase (AChE) activity by plant extracts was measured by the micro-plate assay adapted from Ellman's colorimetric method described by **ELDEEN et al. (2005)**. Buffers used in the assay were prepared as follows: Buffer A - 50 mM Tris-HCl, pH 8.0; Buffer B - 50 mM Tris-HCl, pH 8.0, containing 0.1% bovine serum albumin (0.5 g BSA in 500 ml buffer A); Buffer C - 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, 0.02 M $MgCl_2$**.**$6H_2O$ (2.92 g NaCl and 2.03 g $MgCl_2{\cdot}6H_2O$ in 500 ml buffer A).

In brief, sample extracts (25 µl, 10 mg ml^{-1}) were added to millipore sterile water (25 µl) in a 96-well microtitre plate and serially diluted. Subsequently, acetylthiocholine iodide (ATCI) (25 µl, 15 mM), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in buffer C (125 µl, 3 mM) and buffer B (50 µl) were added in sequence. Absorbance was measured at 405 nm using an Opsys MR^{TM} microplate reader every 45 s (three times). Acetylcholinesterase (25 µl, 0.2 U ml^{-1}) was then added and absorbance read every 45 s (five times). The rate of the enzyme reaction was calculated. Inhibition of acetylcholinesterase was calculated by comparing the rates of enzyme reactions of sample extracts to that of the blank (water). Galanthamine, at a final concentration in the assay of 20 µM was used as a positive control. The experiment was done in duplicate, and AChE percentage inhibition was calculated by using the equation:

Inhibition (%) $=1-\left[RR_{sample}\ /RR_{control}\ \right]\times 100$ (Equation 6.2)

where RR_{sample} is the rate of reaction of the sample extracts and $RR_{control}$ is that of the blank (water).

Calculation of IC_{50} values was done using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., USA).

6.2 RESULTS AND DISCUSSION

6.2.1 ANTIBACTERIAL ACTIVITY

The results of the antibacterial assay (MIC and MBC) are presented in Table 6.1. Extracts of *S. birrea* showed high bioactivity against the Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). The highest activity against Gram-positive bacteria (MIC = 0.098 mg ml^{-1}) was recorded for *S. birrea* young stem extracts against *B. subtilis*. The highest activity (MIC < 1.0 mg ml^{-1}) against Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) was achieved with ethanolic fractions of *S. birrea* young stem and leaf extracts. Gram-negative bacteria are more resistant and impermeable to certain antibacterial compounds compared to Gram-positive bacteria due to the complex and unique multilayered organisation of the cell wall which is composed of lipopolysaccharides and a peptidoglycan-containing matrix **(RISCO and PINTO DA SILVA 1998)**. This structural feature may explain the reported difference in sensitivity to plant extracts between Gram-positive and Gram-negative bacteria **(RABE and VAN STADEN 1997; ELDEEN et al. 2005)**. The MIC values of the different extracts were translated to total antibacterial activity by dividing the quantity extracted per gram by the MIC value **(ELOFF 2001)**. Based on the most susceptible bacterial strain, ethanolic extracts were the most active in the order, young stem extracts (1609.1) > leaf extracts (792.8) > opercula extracts (21.6 ml g^{-1}) (Table 6.1). For example, the bioactive compounds present in 1 g of *S. birrea* young stem extracts would still inhibit *Bacillus subtilis* growth when diluted to 1609.1 ml. The potency of *S. birrea* young stem extracts was 2 times that of leaf extracts. In contrast, **ELOFF (2001)** reported that the total activity of *S. birrea* leaf extracts was 11 times less active than stem bark extracts. The present results suggest that *S. birrea* leaves can be used effectively for the treatment of bacterial ailments. In the bactericidal evaluation, ethanolic fractions of *S. birrea* young stems,

leaves, and opercula were most active (MBC = 1.56 mg ml^{-1}) against Gram-positive bacteria.

6.2.2 ANTIFUNGAL ACTIVITY

Antifungal activity of the plant extracts was evaluated using *C. albicans* and the obtained MIC and MFC values are presented in Table 6.2. The therapy of fungal infections caused by opportunistic pathogens such as *C. albicans* remains a major medical challenge **(SANGETHA et al. 2009)**. Infection by *C. albicans* leads to the formation of a biofilm which is resistant to the penetration of antifungal agents **(SANGETHA et al. 2009)**. The most active extract was *S. birrea* young stem ethanolic fraction (MIC = 1.56 mg ml^{-1} and MFC = 1.56 mg ml^{-1}). Leaf and opercula ethanolic extracts recorded MIC and MFC values of 3.125 mg ml^{-1} against *C. albicans*. Based on total activity (ml g^{-1}), ethanolic fractions had the highest potency against *C. albicans* in the order, *S. birrea* young stem extracts (101.0) > *S. birrea* leaf extracts (98.9) > opercula extracts (5.4). The results indicate that *S. birrea* leaves are as effective as young stem extracts. Almost 20 times the dose of opercula extracts is required for an equal effect as young stem extracts against *C. albicans*. Dichloromethane extracts had comparable MIC values (3.125 mg ml^{-1}) to ethanolic extracts. However, except for leaf extracts (MFC = 3.125 mg ml^{-1}), DCM fractions of young stems and opercula extracts had comparatively low potency (MFC = 6.250 mg ml^{-1}). Petroleum ether extracts exhibited the lowest potency against *C. albicans* (MIC = 6.250 mg ml^{-1}) for leaves, young stems and opercula. The observed antifungal results against *C. albicans* suggest that phenolic compounds in the extracts are responsible for the bioactivity. Using an ultrastructural approach, **SANGETHA et al. (2009)** showed that *Cassia spectabilis* leaf extract prevented formation of a *C. albicans* biofilm at an MIC of 6.25 mg ml^{-1}. Findings of the present study suggest that *S. birrea* leaf and young stem extracts may be functioning by interfering with the formation of a *C. albicans* biofilm.

TABLE 6.1 *In vitro* antibacterial activity of *S. birrea* crude extracts

Plant part	Solvent	Extract Yield (mg g^{-1})	MIC (mg ml^{-1})				Total activity (ml g^{-1})[c]	MBC (mg ml^{-1})			
			B. s [a]	*S. a*	*E. c*	*K. p*		*B. s*	*S. a*	*E. c*	*K. p*
Leaves	PE	30.4	3.125	3.125	3.125	6.25	9.7	12.5	12.5	12.5	12.5
	DCM	9.0	3.125	3.125	6.25	6.25	2.8	3.125	6.25	6.25	6.25
	EtOH	309.2	**0.39**[b]	**0.39**	**0.39**	**0.78**	792.8	1.56	1.56	3.125	3.125
Young stems	PE	2.7	**0.39**	**0.39**	1.56	1.56	6.9	1.56	3.125	6.25	3.125
	DCM	14.6	**0.39**	**0.78**	3.125	1.56	37.4	3.125	3.125	6.25	6.25
	EtOH	157.7	**0.098**	**0.195**	**0.39**	**0.39**	1609.1	1.56	1.56	3.125	6.25
Opercula	PE	1.8	**0.39**	**0.39**	1.56	1.56	4.6	6.25	3.125	3.125	3.125
	DCM	1.5	1.56	**0.78**	3.125	1.56	1.9	3.125	3.125	3.125	6.25
	EtOH	16.9	**0.78**	**0.78**	1.56	1.56	21.6	1.56	1.56	6.25	6.25
Neomycin (µg ml^{-1})			0.195	0.78	6.25	1.56		3.125	3.125	6.25	6.25

[a] *B. s = Bacillus subtilis; S. a = Staphylococcus aureus; E. c = Escherichia coli; K. p = Klebsiella pneumoniae*.

[b] Extracts with values in bold font are considered very active (MIC < 1.0 mg ml^{-1}).

[c] Total activity was calculated for each plant extract based on the bacterial strain with the lowest MIC value.

TABLE 6.2 *In vitro* antifungal activity against *C. albicans* of *S. birrea* crude extracts

Plant part	Solvent	MIC (mg ml^{-1})	MFC (mg ml^{-1})	Extract yield (mg g^{-1})	Total activity (ml g^{-1})
Leaves	PE	6.250	6.250	30.4	4.8
	DCM	3.125	3.125	9.0	2.8
	EtOH	3.125	3.125	309.2	98.9
Young stems	PE	6.250	6.250	2.7	0.4
	DCM	3.125	6.250	14.6	4.6
	EtOH	1.560	1.560	157.7	101.0
Opercula	PE	6.250	6.250	1.8	0.2
	DCM	3.125	6.250	1.5	0.4
	EtOH	3.125	3.125	16.9	5.4
Amphotericin B (µg ml^{-1})		4.82×10^{-2}	1.93×10^{-1}		

Total activity was calculated based on the MIC values.

6.2.3 CYCLOOXYGENASE ENZYME INHIBITION

The bioactivity of sample extracts in the inflammation process was evaluated on their ability to inhibit COX-1 and COX-2 enzymes which are involved in the biosynthesis of prostaglandins. Cyclooxygenase, which exists as the constitutive (COX-1) and induced (COX-2) isoforms, is the principal enzyme in the synthesis of prostaglandins from arachidonic acid **(BOTTING 2006)**. Prostaglandins are lipid mediators that elicit biological effects such as pain, fever and inflammations **(BOTTING 2006)**. Results on percentage inhibition of COX-1 and COX-2 enzymes by sample extracts at 250 µg ml^{-1} in the assay are presented in Table 6.3. All extracts and fractions showed high COX-1 enzyme inhibition (90.7-100%). In particular, the COX-1 inhibitory effects of ethanolic extracts were very high (100%) in sharp contrast to their non-activity against the COX-2 enzyme. Petroleum ether and dichloromethane fractions exhibited high inhibition against COX-2 enzyme (77.7-92.6%). Extracts that

showed high activity (≥ 70%) in the COX-2 assay were evaluated at lower doses (125 and 62.5 µg ml^{-1}) against both cyclooxygenase enzymes. Fig. 6.1 depicts the dose-dependent prostaglandin synthesis inhibition (%) when evaluated against COX-1 enzyme. A similar relationship is presented in Fig. 6.2 for COX-2 enzyme. Both Fig. 6.1 and Fig. 6.2 show an increase in prostaglandin synthesis inhibition with increasing concentration of the plant extracts.

The high COX-1 inhibitory activity by ethanolic extracts could be a false-positive effect due to the presence of tannins which bind to enzymes resulting in the inhibition of prostaglandin synthesis **(ELDEEN et al. 2005)**. *Sclerocarya birrea* is traditionally used in the treatment of several ailments including snake and scorpion bites, fever, pain and inflammation conditions **(BRACA et al. 2003)**. Other Anacardiaceae species, such as *H. caffrum* are also used in the alleviation of pain, among other conditions **(JÄGER et al. 1996; BUWA and VAN STADEN 2007)**. The results of the anti-inflammatory assay have shown that non-polar compounds of *S. birrea*, extracted using PE and DCM, are more active compared to ethanolic polar constituents in the prostaglandin biosynthesis pathway against COX-2 enzyme. Based on COX-2 inhibition, the highest potency was in the order young stem extracts > leaf extracts > opercula extracts for the DCM fraction, and young stems > opercula > leaves for the PE fraction. Therefore, compared to young stems, leaves are equally effective as sources of anti-inflammatory agents.

The high activity of the non-polar extracts is likely due to lipophilic compounds, which are active in low concentrations arising from their superior resorption through the cell membrane **(ZSCHOCKE and VAN STADEN 2000)**. Specific cyclooxygenase inhibitors acting on their own or in synergism with other active compounds in the extracts are responsible for the anti-inflammatory activity **(ZSCHOCKE et al. 2000a)**. The inactivity of the polar extract against COX-2 enzyme suggests some specific interference in the biosynthesis pathway. **ZSCHOCKE et al. (2000a)** showed that phenolic compounds extracted by polar solvents interfere in enzyme bioassays through unspecific binding phenomena. The inactivity may also be due to bound proanthocyanidins which are known to form insoluble complexes with proteins including enzymes. Furthermore, negative results do not necessarily mean that the

extracts are without anti-inflammatory activity, as the active compound(s) could work at alternative sites in the complex inflammation process **(JÄGER et al. 1996)**.

TABLE 6.3 COX-1 and COX-2 enzyme inhibitory activity of *S. birrea* crude extracts

Plant part	Inhibition (%)					
	COX-1			COX-2		
	PE	DCM	EtOH	PE	DCM	EtOH
Young stems	95.9 ± 2.06	93.6 ± 2.14	100 .0± 0.00	92.6 ± 1.23	92.1 ± 4.21	0.0 ± 0.00
Leaves	90.7 ± 4.81	95.4 ± 1.93	100 .0± 0.00	81.6 ± 4.01	89.7 ± 2.43	0.0 ± 0.00
Opercula	91.7 ± 3.09	92.5 ± 0.89	100 .0± 0.00	82.1 ± 2.43	77.7 ± 2.52	0.0 ± 0.00
Indomethacin	67.1 ± 2.74			67.1 ± 2.62		

Prostaglandin synthesis inhibition by indomethacin was evaluated at concentrations of 5 µM and 200 µM for COX-1 and COX-2, respectively.

Percentage inhibition of prostaglandin synthesis is expressed as means ± SEM (n = 4).

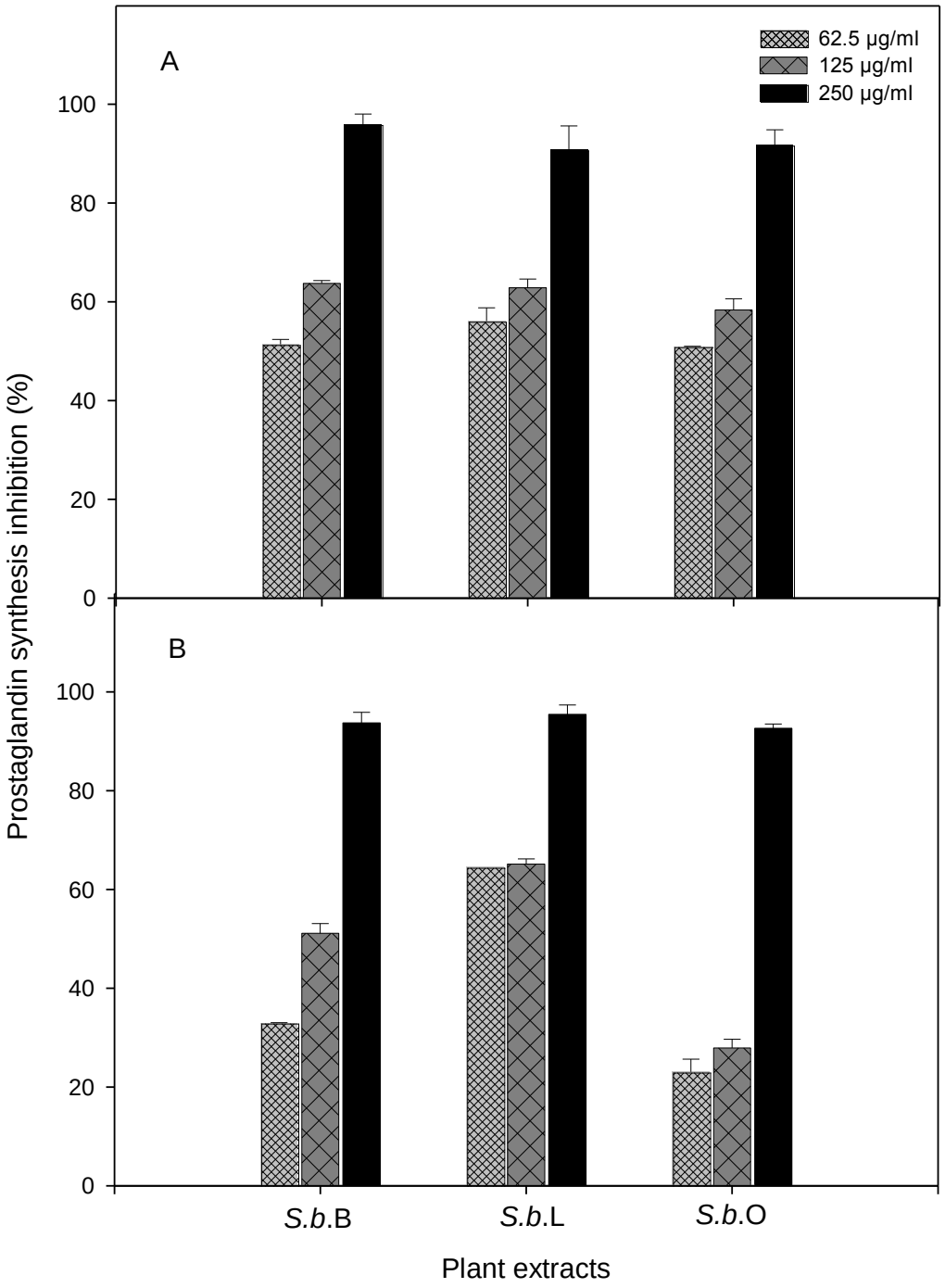


FIGURE 6.1 Dose-dependent prostaglandin synthesis inhibition (%) of *S. birrea* plant extracts on COX-1 enzyme. **(A)** Petroleum ether extracts and **(B)** dichloromethane extracts. *S.b*.B, *S. birrea young* stem; *S.b*.L, *S. birrea* leaf; *S.b*.O, *S. birrea* opercula. Inhibition by indomethacin was 67.1 ± 2.74%.

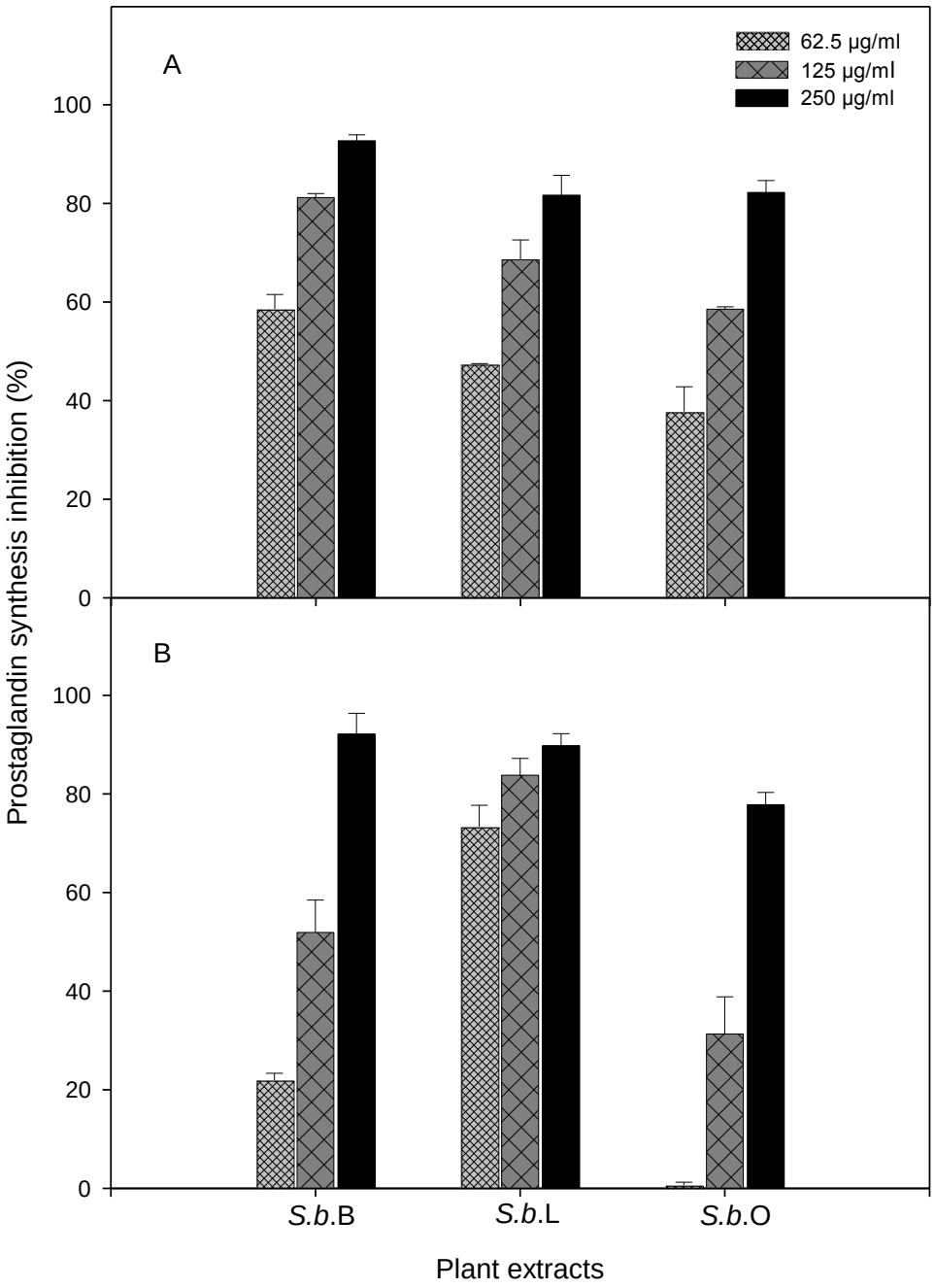


FIGURE 6.2 Dose-dependent prostaglandin synthesis inhibition (%) of *S. birrea* plant extracts on COX-2 enzyme. **(A)** Petroleum ether extracts and **(B)** dichloromethane extracts. *S.b*.B, *S. birrea* young stem; *S.b*.L, *S. birrea* leaf; *S.b*.O, *S. birrea* opercula. Inhibition by indomethacin was 67.1 ± 2.62%.

6.2.4 ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

Results of the AChE inhibitory activities (%) of the sample extracts are presented in Fig. 6.3. *Sclerocarya birrea* plant extracts inhibited acetylcholinesterase in a dose-dependent manner for the dichloromethane and methanol fractions suggesting a possible therapeutic value. The highest inhibition at 0.5 mg ml^{-1} for the dichloromethane fraction was 59.0 ± 0.93% for *S. birrea* young stem extracts. The methanol fraction yielded the highest AChE inhibitory activity of 73.8 ± 17.98% for *S. birrea* young stem extracts. The petroleum ether fraction showed low AChE inhibitory activity even at the highest tested concentration of 0.5 mg ml^{-1} for all *S. birrea* plant extracts. The highest inhibition for the petroleum ether fractions was 38.8 ± 5.77% for leaf extracts. The results suggest that lipophilic compounds of *S. birrea* are not involved in the acetylcholinesterase inhibition process. The IC_{50} values of the sample extracts indicating the AChE inhibitory activity are presented in Table 6.4. The IC_{50} values (mg ml^{-1}) for dichloromethane extracts ranged from 0.1053 (leaf extracts) to 0.3519 (opercula extract). Methanol extracts had IC_{50} values ranging from 0.478 mg ml^{-1} (young stems) to 4.145 mg ml^{-1} (leaves). The IC_{50} value for the positive control, galanthamine, was 0.37 µM.

Plant secondary metabolites possess a range of pharmacological potencies including acetylcholinesterase inhibitory effects **(ELDEEN et al. 2005; DOHI et al. 2009)**. Acetylcholinesterase (AChE), the principal enzyme in the hydrolysis of acetylcholine is involved in the development of Alzheimer's disease (AD). Alzheimer's disease is a progressive age-related disorder that is characterised by the degeneration of neurological function manifested in the loss of cognitive ability, loss of reasoning ability, agitation and general behavioural dysfunction **(FELDER et al. 2000)**. According to the cholinergic hypothesis, the replacement of acetylcholine which is progressively lost during the progression of AD delays the loss of cognitive ability **(FELDER et al. 2000)**. Acetylcholinesterase inhibitors function by increasing acetylcholine within the synaptic region thereby restoring deficient cholinergic neurotransmission **(FELDER at al. 2000)**. People who rely on the traditional health system may derive some benefit through the use of medicinal plant species in managing the adverse effects of conditions such as Alzheimer's disease. One of the early symptoms of Alzheimer's disease is neuroinflammation, accompanied by the

upregulation of COX-2 enzyme at the inflammation sites **(HOOZESMANS et al. 2006)**. *Sclerocarya birrea* plant extracts exhibit high anti-inflammatory activity, suggesting a possible role in the control of neuroinflammation in the early pathological progression of Alzheimer's disease.

TABLE 6.4 Inhibition of AChE activity (IC_{50}) by *S. birrea* crude extracts

Plant part	IC_{50} (mg ml^{-1})		
	PE	DCM	MeOH
Young stems	ND	0.1585	0.478
Leaves	ND	0.1053	4.145
Opercula	ND	0.3519	2.384

ND, not determined, represents extracts with a maximum inhibition below 50% at the highest tested concentration of 0.5 mg ml^{-1}.

The IC_{50} value for the positive control, galanthamine, was 0.37 µM.

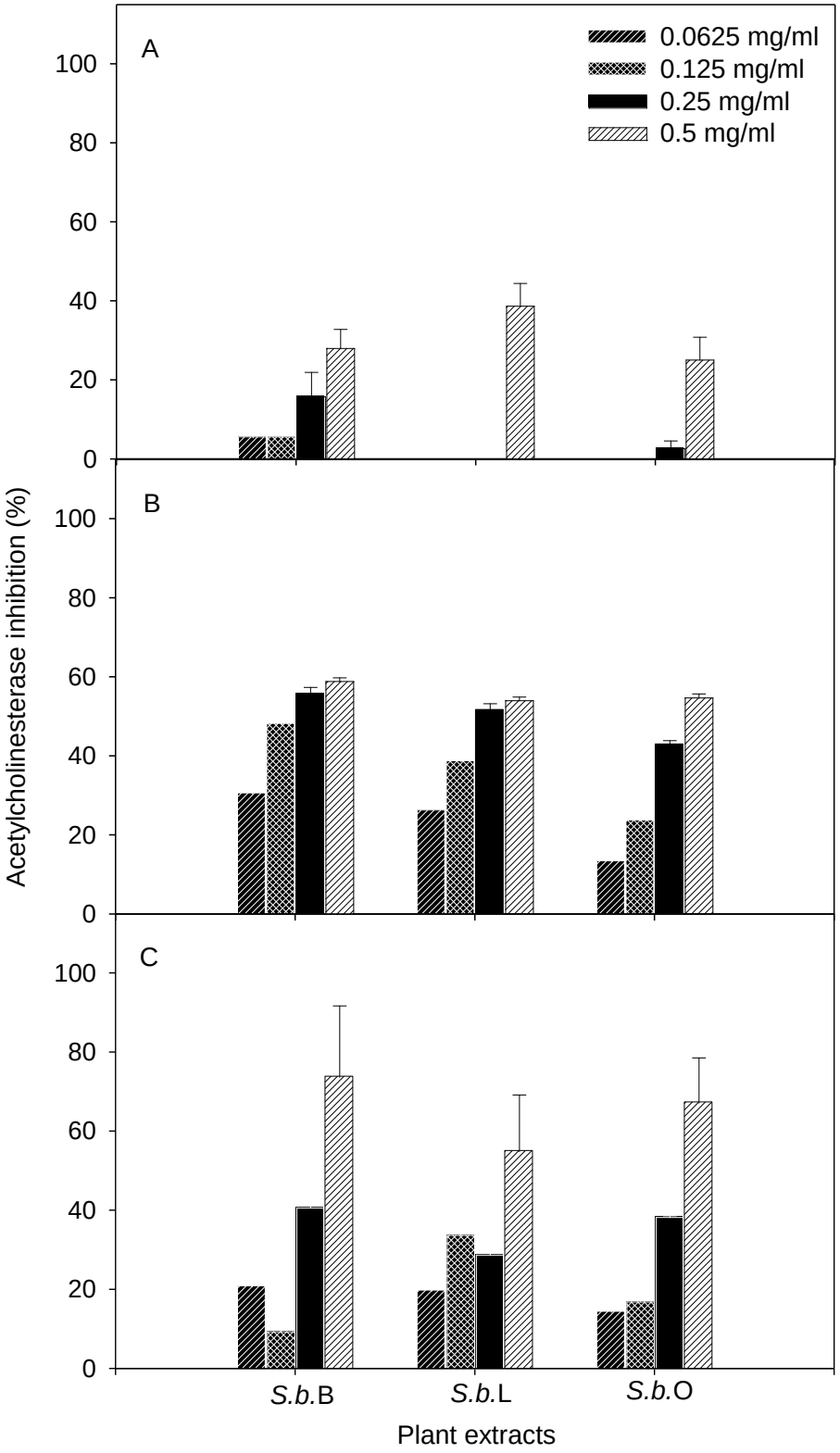


FIGURE 6.3 Dose-dependent AChE inhibitory activity (%) of plant extracts. **(A)** Petroleum ether fraction, **(B)** dichloromethane fraction, and **(C)** aqueous methanol fraction. *S.b*.B, *S. birrea* young stems; *S.b*.L, *S. birrea* leaves; *S.b*.O, *S. birrea* opercula.

6.3 CONCLUSIONS

Plant part substitution as suggested by **ZSCHOCKE et al. (2000b)** and **JÄGER and VAN STADEN (2005)** can be a practical strategy for the conservation of medicinal plants. Based on the demonstrated high antimicrobial and anti-inflammatory, and moderate acetylcholinesterase inhibitory bioactivities of renewable plant parts of *S. birrea*, non-destructive harvesting of this species may be possible. Though *S. birrea* is at present neither extinct nor threatened, its multipurpose uses in traditional medicine renders it highly vulnerable to over-exploitation, hence the need to protect the available wild populations for its future regeneration.

CHAPTER 7
GENERAL CONCLUSIONS

Plants have been central to the sustenance and survival of mankind throughout human existence. People have relied on plants for numerous products including medicine and nutrition. However, the increasing human population continues to exert pressure on the plant resource base, thereby threatening its sometimes fragile biodiversity. To date some plant species have become extinct whilst numerous others are under threat, due to over-exploitation and expansion of agricultural lands into their natural habitats. Throughout human existence some elite plant genotypes have been systematically selected and cultivated to meet the growing demand of an ever increasing population. Recently, advances in plant sciences have hastened the development of new cultivars using the diverse genetic pool of wild species. The Anacardiaceae which comprises 73 genera and 600 species constitute an important family of plants in the tropical and sub-tropical regions. Some Anacardiaceae species namely, *A. occidentale*, *M. indica*, *P. vera*, and *S. birrea*, have great social and economic significance both as medicinal and nutritional plants. These plants are unique in their wide climatic adaption including the arid and semi-arid regions. Apart from *S. birrea*, the other three species have been brought into conventional cultivation. In recent times, research into the application of plant biotechnology has increased for *A. occidentale, M. indica* and *P. vera*, whilst remaining stagnant for *S. birrea* despite its enormous potential (refer to Table 1.2).

The cultivation of marula has been hampered by the limited scope of traditional propagation techniques. Seed propagation is known to be associated with high levels of genetic variability which is undesirable in plant production, although beneficial in terms of biodiversity conservation of species. Plant improvement and large scale commercial propagation would require plant tissue culture techniques **(TIKAT and ONAY 2009)**. Apart from offering a possible solution to challenges of clonal propagation of elite varieties **(TILKAT and ONAY 2009)**, micropropagation also provides an option for conservation of plant species. For multipurpose tree species such as marula, for which the mature tree bark and roots are preferred in traditional medicine, another conservation strategy could be plant part substitution.

The present study sought to explore the *in vitro* propagation of *S. birrea* and provide the basis for future research in the application of plant biotechnology; and to investigate the secondary metabolite profiles of its renewable plant parts as alternative sources of medicinal remedies, thereby providing options in its conservation.

An *ex vitro* seed germination study indicated that after-ripening and cold stratification are critical factors. Cold stratification (5 °C) of marula nuts for 14 days improved germination (65%) as compared to non-stratified nuts (32%). However, the genetic variability arising from seed propagation renders it undesirable as a source of planting material for the perpetuation of desirable traits. Direct shoot organogenesis was achieved through the induction of leaf-derived nodular meristemoids on MS basal medium and WPM supplemented with BA in combination with NAA, IBA or IAA. Induction of nodular meristemoids from 86% of the leaf cultures was achieved on MS medium with 4.0 µM BA and 1.0 µM NAA. High levels (≥ 78%) of induction were also achieved on WPM with different concentrations of BA and IBA. The highest conversion of nodular meristemoids into shoots on MS initiation medium was only 22% for 4.0 µM BA and 1.0 µM NAA. This was improved to 62% when nodular clusters were cultured in MS liquid medium. Plant regeneration through nodule culture has potential for application in mass micropropagation and breeding of *S. birrea*. Future research on leaf-derived nodular meristemoids should focus on clonal propagation of identified elite genotypes from mature trees. The induction of leaf-derived nodular meristemoids has potential for the development of encapsulated synthetic seeds and the application of cryopreservation technology.

The induction of adventitious shoots and roots are important phases in micropropagation. The type and concentration of plant growth regulators used have major influences in the success of these organogenic processes. Three auxins (IAA, IBA and NAA) and four aromatic cytokinins (BA, *m*T, *m*TR, *and mem*TR) were evaluated for their potential to induce adventitious shoot and root formation in *S. birrea* shoots, hypocotyls and epicotyls. Among the evaluated cytokinins, the highest adventitious shoot induction (62%) was achieved on MS medium supplemented with *m*T (8.0 µM). The lowest adventitious shoot induction (2.5%) was obtained on MS

medium containing 2.0 µM *mem*TR. The highest adventitious shoot induction for hypocotyls was 55% on MS medium supplemented with *m*T (8.0 µM). For the tested auxins, IBA induced adventitious rooting in 91% of shoots at a concentration of 4.0 µM after 8 weeks in culture. However, the *in vitro* rooted plants only survived for two weeks when transferred *ex vitro*. The high success in *in vitro* rooting was not translated to the *ex vitro* environment. The failure of *in vitro* plantlets to survive *ex vitro* conditions may have been caused by root morphological deficiencies. The state of development of the roots should be confirmed by light microscopy. To overcome the acclimation challenge, growth of plantlets *in vitro* can be prolonged by a further 30 to 60 days to allow for adequate development of roots. Therefore, acclimation of *in vitro* rooted shoots requires in-depth research to overcome death of the young plants *ex vitro*. The episodic growth pattern that is characteristic of most woody species was observed in the root induction experiments. This problem may be overcome by using 'force-flushed rejuvenated shoots' **(VIEITEZ et al. 2009)**. *In vitro* plantlets growing under a 16-h photoperiod produced the highest stomatal density (number per mm^2) and normal mature stomata.

The phytochemical content (total phenolics, proanthocyanidins, gallotannins, flavonoids), antioxidant, antimicrobial and anti-inflammatory activities of *S. birrea* extracts were evaluated using *in vitro* bioassays. Methanolic extracts of the young stem bark and leaves contained high levels of these phytochemical compounds. *Y*oung stem extracts contained the highest levels of total phenolic content, flavonoids and gallotannins, whereas leaf extracts had the highest concentration of proanthocyanidins. *Sclerocarya birrea* extracts exhibited high antioxidant activities based on the DPPH, ferric-reducing antioxidant power and *β*-carotene linoleic acid assays. In the DPPH radical scavenging assay young stems (EC_{50} = 5.028 µg ml^{-1}) and leaves (EC_{50} = 5.602 µg ml^{-1}) were more potent when compared to ascorbic acid (EC_{50} = 6.868 µg ml^{-1}). In the ferric-reducing antioxidant power assay, young stem extracts showed comparable bioactivity to BHT. The antioxidant activity calculated based on the rate of *β*-carotene bleaching was high for all the extracts (89.6-93.9%). *Y*oung stem and leaf ethanolic extracts exhibited high bioactivity (MIC < 1.0 mg ml^{-1}) against both Gram-positive (*B. subtilis* and *S. aureus*) and Gram-negative (*E. coli* and *K. pneumoniae*) bacteria. Young stem ethanolic extracts were also active

against *C. albicans* (MIC = 1.56 mg ml^{-1}). Moderate AChE inhibition was achieved with the DCM and MeOH fractions of young stems and leaves. The DCM fraction of leaf extracts had the highest AChE inhibitory activity (IC_{50} = 0.1053 mg ml^{-1}). All the tested extracts exhibited high COX-1 inhibition of over 90%. In the COX-2 assay, PE and DCM fractions showed high inhibitory activity that ranged from 77-92%. The tested plant parts provide a source of medicinal secondary metabolites which have highly potent antioxidant, antimicrobial and anti-inflammatory activities, and may be beneficial to the health of consumers. The moderate AChE inhibition may be through the regulation of neuro-inflammation.

The plant tissue culture results obtained in this study provide fundamental insights for future research in micropropagation of *S. birrea*. In particular, nodule culture offers potential prospects in the mass propagation for both plant biotechnology downstream applications and conservation. The high pharmacological activities of the young stems and leaves provide alternatives to mature bark and roots as remedies in traditional medicine. Thus, conservation of *S. birrea* can further be achieved by plant part substitution.

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