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Propagation of *Coleonema album* (Thunb.) Bartl. & J.C. Wendl. *-* a horticultural and medicinal plant

Submitted in fulfillment for the degree of

MASTER OF SCIENCE

By

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STUDENT DECLARATION
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Propagation of *Coleonema album* (Thunb.) Bartl. & J.C. Wendl. - a horticultural and medicinal plant

I, Olufunke Omowumi Fajinmi, 210556317 (student number) declare that:

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Thesis Title: Propagation of *Coleonema album* (Thunb.) Bartl. & J.C. Wendl.- a horticultural and medicinal plant

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

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PUBLICATIONS FROM THIS THESIS

1. **O.O. Fajinmi**, M.G. Kulkarni, J.F. Finnie and J. Van Staden (Submitted)
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   Contribution: Experimental work and writing of the publication was done by the first author, second author provided technical support, under supervision of the last three authors.

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and Development Annual meeting, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg.

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LIST OF ABBREVIATIONS

ABA-   Abscisic acid

BA - N^6-Benzyladenine

Cks-   Cytokinins

DMRT-   Duncan's Multiple Range Test

GA_3-   Gibberellic acid

h-   Hour

IAA-   Indole-3-acetic acid

IBA -   Indole-3-butyric acid

KAR_1-   Karrikinolide

KIN -   Kinetin

MGT-   Mean germination time

Min-   Minute

*m*T -   *meta*-Topolin

*m*TR -   *meta*-Topolin riboside

Me*m*T-   *meta*-Methoxytopolin

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

Me*m*TTHP -   *meta*-Methoxytopolin tetrahydropyran-2-yl

MS-   Murashige and Skoog

NAA -   α-Naphthaleneacetic acid

NaCl-   Sodium chloride

NH_4NO_3-   Ammonium nitrate

PGR-   Plant growth regulator

Rut-   Rutaceae

SW-   Smoke-water

TDZ –   Thidiazuron

TTC –   Tetrazolium chloride

WHO-   World Health Organization

ABSTRACT

Coleonema album is a South African green treasure, endemic to the Cape region and hard to find in other parts of the country. It is ranked among the most highly utilized medicinal plants at present. The plant extracts exhibit a wide range of pharmacological activities due to its bioactive compounds. Immunat, a tincture from this plant is marketed commercially. Medicinal plant gatherers are on the lookout for this plant due to its high demand and market price. Several natural product companies in South Africa are now exporting *Coleonema album* oil. Its showy beautiful white flowers make it a valuable ornamental plant. There is no accredited germination protocol available for this medicinal and ornamental plant till date.

In this study, the chemical and environmental conditions required for optimum seed germination and seedling growth were studied. The effect of light, temperature shifts, cold and warm stratifications, smoke-water, butenolide, pH levels, plant growth regulators (kinetin, 6-benzyladenine, indole acetic acid and α-Naphthalene acetic acid), salt solutions (sodium chloride and ammonium nitrate) on seed germination were investigated. Seeds showed favourable response of germination at low temperatures (10 and 15 ⁰C). Seeds exposed to continuous darkness at low temperatures showed highest germination at 15 ⁰C. Temperature shift (from 20 ⁰C to 15 ⁰C) favoured seed germination. pH 6 is the most conducive pH level for *Coleonema album* seed germination (52.5%). Sodium chloride and ammonium nitrate solutions (pretreatment for 2 weeks) significantly improved germination at a 10^{-2} M concentration with 62.5 and 75% germination respectively. *Coleonema album* seeds exhibit a temperature-dependent physiological dormancy. Seed germination of this plant is quite erratic as seed germination started 21 days after incubation and lasted for over 90 days. Germination rate of the seeds is too slow to meet the local and international demand for this plant.

Plant tissue culture techniques have been used as an alternative for mass cultivation of plants that are difficult to propagate from seeds. Therefore, an effective micropropagation protocol was developed as a measure to conserve this highly utilized

medicinal plant. The effects of the cytokinins: BA, kinetin, TDZ and topolins on *in vitro* shoot formation of *Coleonema album* were investigated. Of all the cytokinins tested, 5 µM *m*T gave the highest number of shoots (14.50) per explant. Combination of 5 µM *m*T with various concentrations of IBA and NAA significantly increased shoot production. Shoots cultured on IBA supplemented medium produced roots. Root production from NAA cultures is unfavourable as most of the shoots developed callus at the base of the stem.

The result of this study has shown that *m*T has potential as a highly active alternative to BA and other cytokinins for *Coleonema album in vitro* shoot production. Over 1,000,000 plantlets could be produced annually using the *in vitro* propagation protocol developed in this study. The results of this study will be useful as a guide for mass cultivation of this ornamental and medicinal plant. It will also provide an opportunity for propagation of *Coleonema album* in other parts of the country. By virtue of this, the pressure on the wild population of this species could be greatly reduced.

CHAPTER 1. INTRODUCTION

Plant availability and abundance is crucial to human existence and survival on earth. Plants nourish the atmosphere with oxygen and offer provision for shelter, food, clothing, raw materials and medicine. Plants have been a major source of medicine even though synthetic drugs have been incorporated into our healthcare system. Man still taps from the myriad of available flora in his environment, especially in poor communities where healthcare facilities are inadequate or unavailable. Pharmaceuticals are prohibitively expensive for most of the world's population, half of which lives on less than two U.S dollars per day **(DASILVA *et al.,* 2002)**. In comparison, medicinal plants can be grown from seeds or gathered from the wild at little or no cost.

1.1. Plants and traditional medicine

The World Health Organization (WHO) estimates that 80% of the world's population uses herbal medicine **(WORLD HEALTH ORGANIZATION, 2002)**. Research has shown that over 40% of plants comprise key ingredients which can be used for prescription drugs. Pharmaceutical companies depend on traditional medicine as their guide. Plants have evolved the ability to synthesize chemical compounds as a defence against attack from a wide variety of predators such as insects and herbivorous mammals **(GOWTHAM *et al.,* 2012)**. According to the authors, some of these compounds whilst being toxic to plant predators are beneficial in treating human diseases. Many of the herbs and spices used by humans to season food also yield useful medicinal compounds **(LAIP and ROY, 2004; TAPSELL *et al.,* 2006)**.

1.2. Africa: an ancient rich culture of herbal medicine

Africa as a continent is known to have a long and impressive history of traditional medicine use which has been shaped by its culture and beliefs. This knowledge is increasing and is being transferred from one generation to the next. Traditional medicine, as a major African socio-cultural heritage, in existence for several hundred years, was once believed to be primitive and wrongly challenged with animosity,

especially by foreign religions, dating back to the colonial days in Africa and subsequently by conventional or orthodox medical practitioners **(ELUJOBA *et al.,* 2005)**. According to the authors, traditional medicine has been brought into focus for meeting the goals of a wider coverage of the present primary health care delivery. The authors stated that traditional medicine is the first-choice healthcare treatment for at least 80% of Africans who suffer from high fever and other common ailments.

In South Africa, the Madagascar periwinkle (*Catharanthus roseus*) was long used as a folk cure for diabetes. *Siphonochilus natalensis* and *Alepedia amatymbica* have long been hawked by the Zulu people for several years **(WEBB and WRIGHT, 1986)**. In Sierra Leone: cooked soup of tropical yams was used to ease uterine pain from child-birth; *Enantia polycis* was used to cure malaria, jaundice and ulcers **(SUMNER, 2000)**. Records of medicinal plant export from different parts of Africa into the European Union and western countries **(GITHENS, 1948)** shows that Africa has an ancient history of medicinal plant use. Some of these plants are now cultivated commercially in Africa for local and export purposes as the knowledge of their medicinal value has increased greatly.

Africa is endowed with a vast resource of medicinal plants. One of the six most significant concentrations of plants in the world is the Cape Floral Kingdom with its distinctive fynbos vegetation **(LIEBENBERG, 2008)**. The Cape Floral Kingdom is the smallest of the world's six Floral Kingdoms **(TAKHTAJAN, 1986)** and is characterised by its high richness in plant species (8700 species) and its high endemism with 68% plant species confined to the Cape Floral Kingdom **(GOLDBLATT and MANNING, 2002)**. Amongst the 10 largest families in the flora is the Rutaceae (Rut) **(GOLDBLATT and MANNING, 2002)**. South African indigenous floral species provides valuable materials for the discovery of novel bioactive chemicals and the development of new drugs from natural origins **(LIEBENBERG, 2008)**. Unfortunately a lot of plants are being harvested indiscriminately due to their high medicinal value and market price.

1.3. Sustainability of South Africa's green heritage

Over-exploitation of medicinal plants poses a threat to availability and sustainability of the South African green heritage. According to **BOTHA *et al.* (2004)**,

the rising demand for medicinal plants has led to increased pressure on wild plant populations. The author concluded that this combined with shrinking habitats is the reason why many species in South Africa are now facing local extinction.

In South Africa, the primary health care needs of a large proportion of the urban and rural population are fulfilled by traditional medicine **(VAN STADEN, 1998)**. According to the author, most of the plants and/or plant parts used by traditional healers are collected in the wild. The author inferred that the increasing population needing plant material has led to severe pressure on the different environments and a serious threat to plant species.

In a quest to protect medicinal plants in South Africa from the risk of extinction, botanical gardens have been established across the country. This method of conservation can only be effective if it is coupled with the provision of adequate plant material to breeders and the medicinal market. The sustainability of medicinal plants depends partly on the use of propagation techniques as a conservation tool to ensure survival of highly utilized species.

1.4. Why conserve plants?

Plants form an important part of day to day life. Among the innumerable reasons for plant conservation are; aesthetic and cultural **(MABBERLEY, 1987; HOYLES, 1991; HYAM and PANKHURST, 1995; MABEY, 1996)**, economic **(TEN KATE, 1995)** and environmental stability **(HUNTER, 1996; BOWES, 1999)**. There is a need to pay more attention to plants which are of commercial, medicinal and horticultural value such as the Rutaceae species. They are widely known in South Africa for their medicinal and horticultural value. Rutaceae species are among the most traded plants in the international herbal medicinal market. Most of the Rutaceae species used as medicine are popularly referred to as *buchu*.

1.5. Rutaceae

The Rutaceae family also known as the citrus family is a large group of about 161 genera and 1815 species (worldwide), with 21 genera and 289 species native to

southern Africa. Several species of the family Rutaceae are popularly used as medicinal plants due to the presence of highly therapeutic components found in them e.g*. Agathosma* species, *Coleonema album*, *Coleonema juniperum*, *Ruta graveolens*, *Aegle marmelos*, *Clausena anisata* to mention a few. The Rutaceae comprise highly traded species (medicinal) locally and internationally. Apart from the high medicinal value of most Rutaceae species, a high proportion of the members are also of high horticultural value. Several research works have been carried out to determine the bioactive compounds present in different Rutaceae species **(BASER *et al.,* 2006a; BASER *et al.,* 2006b; BASER *et al.,* 2006c; BASER *et al.,* 2006d; VILJOEN *et al.,* 2006)**.

1.5.1. The genus Coleonema

Coleonema is a genus of flowering plants in the family Rutaceae. The eight known species are all from the Western Cape Province of South Africa **(PAGE and OLDS, 1997)**. Species present in this genus are:

i. *Coleonema album*
ii. *Coleonema asphalanthoides*
iii. *Coleonema calycinum*
iv. *Coleonema pulchrum*
v. *Coleonema virgatum*
vi. *Coleonema pulchellum*
vii. *Coleonema nubigena*
viii. *Coleonema juniperinum*

They are plants of flimsy appearance and delicate character, with slender branches and narrow, heath-like leaves that are fragrant when brushed or bruised.

1.5.2. Morphology, flowering, distribution and taxonomy of Coleonema album (Thunb.) Bartl. & J.C. Wendl.

The plant is a decorative shrub with dainty, small white flowers and a sweet, honey smell which makes it an ideal garden plant and an interesting specimen for floral arrangements. It is an erect, much-branched and compact shrub which grows to a

height of 2 m. It is finely branched and new shoots develop at the tips of old branches. Branching occurs from the base of the shrub. Bark is greyish-brown, rough with horizontal leaf scars. Leaves are needle-like or linear-oblong, 12-13.5 mm long and 1.3-1.5 mm broad. Oil glands are visible on the reverse side of the leaves. The leaves when crushed have a characteristic sweet smell. Ripe fruits may be found up to the end of November. The fruit is 5-lobed and ripe seeds are ejected by a catapult mechanism **(JODAMUS, 2003)**.

Coleonema album inflorescences are solitary, axillary and crowded at the branch tips. Closed flower buds are pinkish tinged and appear white when open. Flowers are small, white, 6-7 mm in diameter with a dark green disc at the centre, crowded at the branch tips are 5-11 blooms (FIG 1.1). The flowers are carried in such profusion that the bush is a cloud of white when in flower and attracts bees and butterflies. It flowers from May to November **(JODAMUS, 2003)**.

Coleonema album occurs from Saldanha Bay to the Cape Peninsula and as far east as Bredasdorp (FIG 1.2). This *buchu* appears to be very much a coastal plant, able to withstand strong coastal winds. The only population at any great distance from the sea lies at the southern end of the Bredasdorp Mountains about 19 km from the sea. Plants are found growing amongst outcrops of rocks of the Table Mountain Sandstone series or of the underlying Cape granite, but never on the coastal limestone of the Bredasdorp Series. Plants grow at sea level and up to a maximum altitude of 750 m on Table Mountain **(JODAMUS, 2003)**.

The name *Coleonema* is made up from two Greek words meaning sheath and filament, alluding to the fact that in seven of the eight species in the genus the filaments are connate with and enclosed in the claw of a petal **(JANINE and VAN WYK, 1999)**. According to the authors, *Coleonema* was formerly in the genus *Diosma*, a taxonomy system that caused controversy for over a century. **DE CANDOLE (1824)** relegated nearly all the genera in Diosmeae to the synonym of *Diosma.* In the same year, some of the genera recognized previously were maintained and few new generic names were proposed by **BARTLING and WENDLAND (1824)** but it was not widely accepted. All these genera were transferred back to the genus *Diosma* **(DIETRICH, 1839)**. This decision was supported by **STEUDEL (1841)**. **SONDER (1860)** upheld the classification

of **BARTLING and WENDLAND (1824)**. **WILLIAMS (1982)** excluded 164 species from *Diosma*. *Coleonema* was described by **BARTLING and WENDLAND (1824)** in a revision of the Diosmeae and *Coleonema album* was selected as the type species. In a revision of *Coleonema* in the flora *Capensis*, four species of *Coleonema* were recognized **(SONDER, 1860)**. In the last revision of *Coleonema*, eight species were recognized **(WILLIAMS, 1981)** and is generally followed till present.

FIGURE 1.1: *Coleonema album* flowers crowded at the branch tip.
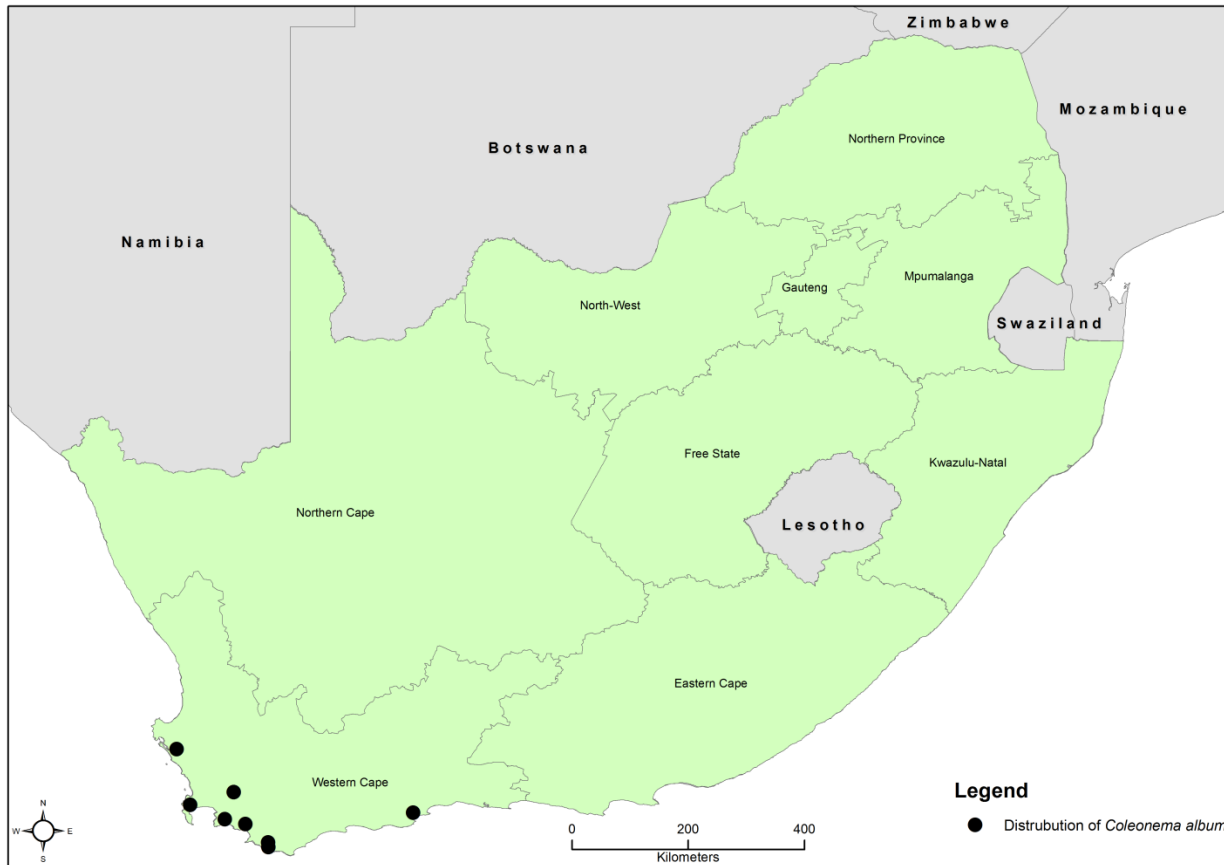
FIGURE 1.2: *Coleonema album* geographical distribution in South Africa (map adopted from http://redlist.sanbi.org/species.php?species=3365-1)

Coleonema album occurs from Saldanha Bay to the Cape Peninsula and as far east as Bredasdorp.

1.5.3. Economic importance, pharmacological activities and medicinal uses of the plant

Coleonema album is of great economic importance. Its oil is clear in color with a fresh herby fragrance and an interesting pepper note. The fragrance is typical of the Cape fynbos. The main chemical components of *Coleonema album* are myrcene, phellandrene, pinene, ocimene, and germacrene-D **(BASER *et al.*, 2006e)**. A wide range of coumarin derivatives have been isolated from *Coleonema album* **(DREYER *et al.*, 1972; GRAY, 1981, GRAY *et al.*, 1986)**. *Coleonema album* plants have huge potential as an ornamental. It forms a cloud of small white flowers during the spring. It is a valuable specimen for floral arrangements. Its showy white flowers and unique aroma makes it of great aesthetic value.

Pharmacological activities of *Coleonema album* are attributed to its terpenes and coumarins (and coumarin derivatives). They have: antithrombotic and antiplatelet **(HOULT and PAYA, 1996),** antimicrobial **(KAYSER and KOLODZIEJ, 1999; LAURIN *et al.*, 1999; LIS-BALCHIN and HART, 2002, OKUNADE *et al.*, 2004; ESTERHUIZEN *et al.,* 2006a; LIEBENBERG, 2008)** antioxidant **(ESTERHUIZEN *et al.,* 2006b LIEBENBERG, 2008; SENTHILKUMAR and VENKATESALU, 2009;)** and analgesic **(LORENZETTI *et al.*, 1991)** activities**.**

A tincture made from *Coleonema album* and marketed as "Immunat" is widely used as an herbal remedy. Some compounds isolated from this plant have activity against several inflammatory mediators **(ELDEEN and VAN STADEN, 2008)**.The plant has a nice scent and can be used as a body deodorant to counter unpleasant smell by direct topical application. It can be used as an alternative to citronella or lemongrass oil around a campfire **(SKIMMELBERG, 2011)**.

1.6. Propagation

Coleonema album produce seeds which are dispersed by an explosive mechanism during the late-spring/summer period. To date, there is no report on germination requirements of *Coleonema album* seeds. Literature has shown that *Coleonema album* is endemic to South Africa and is confined to the Cape region.

However, the contributory effect of the Cape weather conditions on seeds germination and the plant's geographical distribution of the plant are not known.

1.7. Significance of the study

A lot of ethno-botanical work has been done on *Coleonema album* but there is no information available on its seed germination requirements, physiological growth requirement of seedlings and shoot-tip cuttings (*in vitro*). It has been reported that most of the temperate species of Rutaceae exhibit physiological dormancy **(BASKIN and BASKIN, 1998),** indicating that the embryo has low growth potential **(BASKIN and BASKIN, 2004)**. *Coleonema album* is a highly utilized medicinal plant which is gaining popularity locally and internationally. The Red Data List of South Africa shows that *Coleonema album* conservation status is least concerned. However, several natural products in foreign countries are involved in the sale of *C. album* oil. The source of the plant materials from which the oil is derived is not known but there is a possibility that they are purchased from South Africa. If this is the case, *C. album* will soon be critically endangered. Several natural product companies in South Africa are also involved in the production, sale and export of *C. album* oil. The fact that the plant is confined to the Cape region of South Africa calls for concern and action as the medicinal plant markets in all parts of South Africa depends on *C. album* plant materials harvested from the Cape region. A tincture from this plant known as "Immunat" is now marketed commercially. This has led to an increase in demand for *Coleonema album* in the traditional medicine market. Medicinal plant gatherers are on the lookout for this plant due to its medicinal value, high demand and market price. An intensive collection of this medicinal plant from indigenous sources will soon cause a threat to the wild populations unless an effective means of conservation is put in place. Increasing demand for a medicinal plant such as *Coleonema album* can only be met by the establishment of effective propagation protocols. These necessitate research into the seed germination requirements and general physiological growth requirements *in vitro* and *ex situ* as a crucial step to ensure survival of these species and to make available an adequate supply of plant material for breeders and the commercial market. The result of this study

will augment the existing knowledge about this medicinal plant and will serve as an accredited protocol for germination and growth should the plant become vulnerable or endangered as a result of excessive use.

1.8. Aims and objectives of the study

In South Africa *Coleonema album* grows naturally in the Cape region and is hard to find in other parts of the country. The medicinal and horticultural value of this plant makes it an ideal garden plant. Thus, it is necessary to establish an efficient propagation protocol of this medicinal plant. The objectives of this study were to:

- Investigate germination requirements of *Coleonema album* seeds; and
- Determine suitable conditions for *in vitro* propagation of *Coleonema album* plants.

CHAPTER 2. LITERATURE REVIEW

2.1. Seed germination

Seed germination involves the imbibition of water, a rapid increase in respiratory activity, the mobilization of nutrient reserves and the initiation of growth in the embryo **(FENNER and THOMPSON, 2005)**. Some seeds are capable of growth soon after fertilization and, long before their normal harvesting time, while others may be dormant and require an extended resting period or additional development before germination can occur **(COPELAND, 1976)**. Seed germination is an irreversible process, once germination has started; the embryo is committed irrevocably to growth or death **(FENNER and THOMPSON, 2005)**. Seed germination is the resumption of active growth of the embryo that results in the rupture of the seed coat and the emergence of the young plant **(COPELAND, 1976)**. This shows that after the formation and development of the seed it has been in a state of rest during which it is in a relatively inactive state with a low metabolic rate. According to the author, a seed can remain in this state until the time and place are right for resumption of growth.

2.1.1. Seed dormancy, a regulatory mechanism in plant species

Dormancy is most easily observed, measured and defined negatively as the failure of a viable seed to germinate given moisture, air and a suitable temperature for radicle emergence and seedling growth **(AMEN, 1968)**. A dormant seed is viable and has the potential to germinate but requires exposure to certain conditions before germination can occur **(PESSARAKLI, 2001)**. The functions of primary dormancy appear to be two-fold. First, along with the inhibition of germination of developing seeds by the mother plant, primary dormancy helps to prevent precocious germination on the mother plant **(BEWLEY and DOWNIE, 1996)**. Secondly, primary dormancy persists after maturation and shedding, resulting in temporal dispersal of seeds by preventing the immediate and approximately synchronous germination of seeds **(FENNER, 2000)**. The crucial function of dormancy is to prevent germination when conditions are suitable for germination but the probability of survival and growth of the seedling is low

(FENNER and THOMPSON, 2005). The ability of seeds to delay their germination until the time and place are right is an important survival mechanism used by plants to adapt to their environment **(COPELAND, 1976)**. The types of dormancy found in plant species are: physiological, morphological, morpho-physiological, chemical, mechanical, physical and combined dormancy **(BASKIN AND BASKIN, 1998)**.

2.1.1.1. Types of seed dormancy

The different types of dormancy found in plant species have been described by **BASKIN and BASKIN (1998)**. According to the authors, there are seven types of dormancy in seeds of plants.

Physiological dormancy occurs in seeds which are permeable to water, but are dormant as a result of physiological inhibiting mechanisms of the embryo which prevents radicle emergence and growth. Physiological dormancy is the most common type of dormancy found in Rutaceae species **(RIZZINI, 1965; BLOMMAERT, 1972; GARWOOD, 1983; GONZALEZ, 1991; PIERCE and MOLL, 1994; AULD, 2001; OOI, 2007)**. According to **BASKIN and BASKIN (1998),** physiological dormancy prevents germination of freshly matured seeds until seeds are exposed to a narrow range of temperatures. The authors further stated that in some species, physiological dormancy is broken by a relatively short period of cold stratification. In some other species, the dormancy is broken by exposure of seeds to high temperatures and the dormancy is not completely broken or is not broken at all if seeds are cold stratified **(BASKIN and BASKIN, 1986)**.

Morphological dormancy occurs in seeds with underdeveloped and undifferentiated embryos. These seeds need to go through a stage of embryo differentiation, growth and development before germination can occur. In seeds with rudimentary and linear embryos, a large volume of the seed is occupied by endosperm while the embryo may occupy only 1% or less of the seed volume **(NIKOLAEVA, 1969)**. Rudimentary and linear embryos are collectively referred to as underdeveloped embryos **(GRUSHVITZKY, 1967)**. Thus the embryos have to undergo a stage of

growth to a species-specific size and attain maturity before germination can take place. This takes place subsequent to dispersal from the mother-plant.

Morpho-physiological dormancy occurs in seeds which are underdeveloped and at the same time exhibit physiological dormancy. Before this dormancy can be broken, the embryo must grow to a species-specific size **(BASKIN and BASKIN, 1998)** followed by exposure to environmental conditions needed to break physiological dormancy. In some species, the same environmental conditions promote the release of seeds from both dormancy types while other species require different conditions for dormancy release. Some seeds need warm stratification only **(BASKIN and BASKIN, 1990)**, cold stratification only **(BASKIN and BASKIN, 1984c)**, warm followed by cold stratification **(BASKIN and BASKIN, 1984b)** and cold followed by warm followed by cold stratification **(NIKOLAEVA, 1977)**.

Chemical dormancy arises due to the presence of inhibitors in the pericarp. It can be broken by removal of the pericarp or by leaching of the seeds. Some of the inhibitory compounds are either produced in or translocated to the seeds where they block embryo growth. Numerous studies have demonstrated that germination in Petri-dishes is inhibited by a variety of compounds **(EVENARI, 1949; KETRING, 1973)**. One common cause of chemical dormancy in seeds is the presence of abscisic acid (ABA). Levels of ABA in seeds increase during the first half of seed development as the dry weight increase, the levels decrease as the water content of the seeds decline **(HILHORST, 1995)**. High ABA levels in seeds serve to prevent precocious germination of the developing embryo **(ZEEVAART and CREELMAN, 1988)**. ABA prevents germination by inhibiting radicle growth **(KARSSEN, 1976; SCHOPFER _et al.,_ 1979)**. _Crowea, Boronia_ and _Eriostemon_ are members of the Rutaceae and they can be successfully propagated with chemical dormancy-breaking treatments such as leaching and stratification **(MCINTYRE and VEITCH, 1972; WHITEHORNE and MCINTYRE, 1975; ELLIOT and JONES, 1980)**.

Mechanical and physical dormancy arise from the seed's covering structures. Mechanical dormancy results from the presence of a hard, woody fruit wall. The woody structure is usually the endocarp but sometimes the mesocarp also is woody **(HILL, 1933)**. Mechanical dormancy can be overcome artificially by cracking the structure

covering the embryo or naturally by soil organism **(ARTECA, 1996)**. Mechanical dormancy can also be broken by cold stratification **(NIKOLAEVA, 1969, 1977)**. However, stratification did not improve seed germination of *Geleznowia verrucosa* (Rut) **(PAYNTER and DIXON, 1991)**. According to the authors, the removal of a small portion of the testa at or near the radicle end of the seed improved *G. verrucosa* seed germination as the opened testa allowed radicle emergence.

Physical dormancy occurs in seeds as a result of impermeability of seed (or fruit) coats to water. An impermeable seed coat is caused by the presence of one or more layers of impermeable palisade cells. According to **BASKIN and BASKIN (1998)**, a number of physical dormancy breaking techniques have been developed by farmers, horticulturalists, gardeners and researchers. These methods include acid scarification, mechanical scarification, percussion, hot water, dry heat, low temperatures and dry storage. The authors further stated that mechanical and acid scarification treatments will readily make a seed to be permeable to water while the effectiveness of the other methods varies with the species, treatment intensity and duration.

Some seeds exhibit both physical and physiological dormancy. **NIKOLAEVA, (1969)** referred to this type of dormancy as "Combined dormancy" which cannot be broken until the seeds are exposed to conditions that can release it from both types of dormancy. However, in some seeds with combined dormancy, the physiological dormancy is broken before the physical dormancy **(MCKOEN and MOTT, 1984)**.

Dormancy release of seed is highly dependent on the temperature and photoperiod conditions seeds are exposed to during germination. The process of germination starts with water imbibition which is influenced by several factors such as temperature, moisture availability (and its osmotic pressure), permeability of the seed coat, chemical composition of the seed **(COPELAND, 1976)**. However, there are several other factors involved in dormancy release and seed germination. These factors include the plant habitat, soil pH, soil salinity, fire and smoke-water. The induction of germination by the above mentioned factors is dependent on photoperiod and temperature requirements of the seed.

2.1.2. Temperature requirement for germination

The major environmental factor causing changes in dormancy states is temperature, but other factors such as darkness, light, gases, chemicals and water may be important **(BASKIN and BASKIN, 1987)**. These factors can cause changes in dormancy states or prevent germination until dormancy is induced by a change in temperature **(BASKIN and BASKIN, 1998)**. Temperature effects on germination can be expressed in terms of cardinal temperatures which are the minimum, optimum and maximum temperatures at which germination will occur **(COPELAND, 1976)**. The author defined optimum temperature as the temperature giving the highest percentage of germination within the shortest period of time. Much of the confusion about the role of temperature in the induction of dormancy arises from the fact that temperature regulates dormancy and at the same time can act as a germination cue **(FENNER and THOMPSON, 2005)**.

2.1.2.1. Temperature regimes requirement for germination

Temperature is one of the major requirements for seed germination. However, its effect is mainly dependent on the degree and time of exposure. Temperature requirement for dormancy release and germination can be divided into two different effects: constant and alternating temperatures **(FENNER and THOMPSON, 2005)**. According to the authors, in many species germination is reduced or totally inhibited at constant temperatures. Alternating temperatures usually are more favourable for germination than constant ones **(STEINBAUER and GRIGSBY, 1957; MATUMURA *et al.*, 1960; THOMPSON and GRIME, 1983)**. Some species will germinate only at alternating temperature regimes **(GOEDERT and ROBERTS, 1986; PEGTEL, 1988)**. Stimulation of germination by alternating temperatures is frequently common to relatively small seeded species **(FENNER and THOMPSON, 2005)**. **PONS and SCHRODER (1986)** reported that for a high percentage of seeds to germinate, the difference between high and low temperatures must be 10 ºC or more. Irrespective of how alternating temperature cycles are maintained, the choice of regimes to use needs to be based on temperatures occurring in the species habitat **(BASKIN and BASKIN,**

1998). In winter annuals, dormancy release occurs during a progressive reduction in the minimum temperature requirement for germination **(FENNER and THOMPSON, 2005)**. Hence, some winter annuals respond to temperature shifts in a controlled environment. In many cases, the sensitivity of seed populations to alternating temperatures may be influenced by other environmental factors, particularly light **(TOOLE and KOCH, 1977; ROBERTS and BENJAMIN, 1979; TOTTERDELL and ROBERTS, 1980; PROBERT *et al.*, 1986)**. Temperature fluctuations can break seed dormancy of many species **(FENNER, 2000)**. Seed dormancy of some species has been broken by a progressive increase or decrease of temperature in their natural habitat. Thus, a high germination percentage can be attained during germination trials in a controlled environment by the use of temperature-shifts. For example, *Geleznowia verrucosa* germination was improved by increasing temperature under which seeds were incubated from 15/10°C to 20/15 °C **(PAYNTER and DIXON, 1991)**.

2.1.3. Light requirement for germination

As dormancy loss progresses, the rate of germination increases **(ALLEN *et al.*, 1995)** and seeds of some species loose their light requirement for germination **(CORBINEAU *et al.*, 1992)**. Light can prevent the germination of negatively photoblastic seeds **(BASKIN and BASKIN, 1998)**. Reports have shown that light requirement for seed germination varies from one species to the other. Some seeds will germinate under continuous darkness conditions only while others require light for germination.

In studying light requirement for germination, it is crucial to test seeds in light and darkness when they are freshly matured and at regular intervals during the dormancy breaking period **(BASKIN and BASKIN, 1998)**. According to the authors, seed light requirement for germination may change as they come out of dormancy **(BASKIN and BASKIN, 1998)**. Seeds held at dry storage for several months at room temperatures may lose their light requirement for germination **(AMRITPHALE *et al.*, 1984; VIANA and FELIPPE, 1986, 1989; LIMA and FELIPPE, 1988)**. The light requirement for germination may vary with temperature **(BASKIN and BASKIN, 1998)**.

2.1.4. Influence of temperature on seeds sensitivity to photoperiod

The interaction between light and temperature requirement varies between species **(FENNER and THOMPSON, 2005)**. A seed light requirement for germination can be replaced by chilling or overwintering in some species **(FENNER, 2000)**. According to **FENNER and THOMPSON (2005)**, light can substitute totally for alternating temperatures while in some cases the effect of light is merely to reduce the degree of alternation necessary to stimulate germination. Dormancy of many light requiring seeds is also broken in darkness by chilling or alternating temperatures **(BEWLEY and BLACK, 1994)**. Light can substitute for a fluctuating temperature requirement in many species **(FENNER, 2000)**. A regime of alternating temperatures partially suppressed the light requirement and improved germination of *Withania somnifera* seeds **(KAMBIZI *et al.*, 2006)**. High temperatures removed the light requirement for the germination of *Hygrophila auriculata* seeds **(AMRITPHALE *et al.*, 1989)**. Low winter temperatures removed the light requirement for the germination of *Picea mariana* **(FARMER *et al.*, 1984)**.

2.1.5. Plant habitat's influence on seed germination

There is a high level of endemism of Rutaceae species in the limestone flora of South African lowland fynbos **(WILLIS *et al.*, 1996)** such as the Hamansdorp and Agulhas plain of Southern Africa **(COWLING *et al.*, 1992)** and heaths, woodland and forests of Sydney region of South-Eastern Australia **(AULD, 2001)**. The objective of germination ecology is to understand factors responsible for seed germination in a natural habitat **(BASKIN and BASKIN, 1998)**. Thus, to have a successful and high germination percentage in a controlled environment, it is of utmost importance to study the environmental and chemical condition of the natural habitat. The trend these conditions take throughout the year might give a clue as to why and how the seeds germinate during a particular season.

2.1.5.1. Temperature and light requirements for germination in relation to plant habitat and geographical distribution

The response to temperature depends on the species, variety, growing region and duration of time from harvest **(COPELAND, 1976)**. Seeds of winter annuals must be exposed to high summer temperatures for several months to germinate at autumn temperatures in autumn **(ROBERTS and NEILSON, 1982; BASKIN and BASKIN, 1984a; STANDIFER and WILSON, 1988)**. Seeds of most obligate or facultative winter annuals mature in spring and/or early summer and are either dormant or conditionally dormant. These dormant seeds will germinate only at low temperatures (5, 10, 15, 15/6, and 20/10 °C) which do not occur in the habitat in the late spring or summer **(BASKIN and BASKIN, 1983)**. Seed requirements of high summer temperature for germination can be replaced by several months of dry storage at room temperatures **(BASKIN and BASKIN, 1971a, 1982)**. Seeds need to be incubated at alternating temperatures close to those occurring in the plant natural habitat **(BASKIN and BASKIN, 1998)** before germination can take place. Alternating temperatures interact with other environmental factors including light **(PROBERT *et al.*, 1986; VOESENEK *et al.*, 1992)** and nitrates **(VINCENT and ROBERTS, 1977; WILLLIAMS, 1983)** to control germination.

Plants with a wide geographical distribution can germinate under a wide range of temperatures. For example, *Clausena excavata* (Rut) grows in diverse habitats and its seeds germinated at temperatures between 20 and 35 °C in a controlled environment **(VIEIRA *et al.*, 2010)**. Plants with a restricted geographical distribution may require a narrow range of temperatures for seed germination. Winter annuals have a narrow range of temperatures at which their seeds germinate.

The ability to detect light (or its absence) is of great importance for survival **(FENNER and THOMPSON, 2005)**. According to the authors, if a seed that is lying in darkness below the soil germinates; its shoots may not be able to reach the soil surface. The authors further stated that this problem is greatest in small seeds. A small amount of light penetrates a few millimetres from the soil surface **(BLISS and SMITH, 1985; TESTER and MORRIS, 1987)**. Presence of translucent particles such as quartz grains in sand may transmit light **(FENNER and THOMPSON, 2005)**. According to the authors,

in a species that germinate in the dark, there is usually a minority of individual seeds that are light-sensitive.

2.1.5.2. Soil pH and seed germination

Seeds of many species germinate to high percentages over a wide range of pH values **(STUBBENDIEK, 1974; SINGH *et al.*, 1975; MABO *et al.*, 1988; RIVARD and WOODARD, 1989; ARTS and VAN DER HEIJDEN, 1990)**, but those of others germinate to high percentages only at a specific pH. The optimum pH for the germination of *Calluna vulgaris* is 4.0 **(POEL, 1949)**, but it is 7.0 for seeds of *Bidens biternatum* **(AHLAWAT and DAGAR, 1980)** and 8.0 for *Tridax procumbens* **(RAMAKRISHNAN and JAIN, 1965)** and *Euphorbis thymifolia* **(RAMAKRISHNAN, 1965)**. Seeds of *Paulownia tomentosa* did not germinate at pH of 1.5 to 3.5, but they germinated to 79 and 98% at pH 4.0 and 7.0 respectively **(TURNER *et al.*, 1988)**.

2.1.5.3. Does soil salinity of plant habitat inhibit or promote seed germination?

Salinity percentage of sodium chloride (NaCl) in coastal salt marshes ranges from 0.8 to 2.4% **(BEEFTINK, 1977)**. Inland regions with low rainfall and high rates of evaporation accumulate soluble salts in the upper horizons of the soil **(WAISEL, 1972)**. Sodium is one of the most important cations in these salts and one of the most important anions is chloride **(BLACK, 1968)**. High soil salinity has been reported to inhibit seed germination of several plant species. Soil salinity reduce seed water uptake by decreasing the external osmotic potential of the seed **(BEWLEY and BLACK, 1943)**. According to the authors, inhibitory concentrations of sodium chloride on germination vary from one species to another and seed sensitivity to NaCl depends on temperature. The authors further explained that the ability of seeds to germinate at relatively high salinity is determined by salt tolerance of the seeds during the germination stage and also the capacity to remain viable during imbibition under saline conditions. The authors highlighted that some species overcome inhibition of germination by high concentrations of NaCl after transfer onto water which indicates that there is no permanent ion toxicity.

On wet days, salt content of the soil may be diluted by rain **(FENNER and THOMPSON, 2005)**. The timing of seed germination in a saline habitat might be expected to coincide with periods when the soil water is diluted by rainfall and perhaps combined with a prevailing cooler temperature which would reduce evaporation **(FENNER and THOMPSON, 2005)**. Pre-treatment with NaCl stimulated further germination of some plant species in fresh water **(BEWLEY and BLACK, 1943)**.

2.1.5.4. Is fire and smoke-water inhibitory or stimulatory for seed germination?

Smoke-water (SW) and the novel compound from smoke, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (karrikinolide, KAR_1) which is also referred to as butenolide have been used to improve seed germination of several medicinal plants. For example, seed germination of *Aloe ferox* **(BAIRU *et al.,* 2009)**, *Alepedia amatymbica* and *Alepedia natalensis* **(MULAUDZI *et al.,* 2009)** significantly improved with the use of SW. Smoke has been reported to break seed dormancy of *Geleznowia verrucosa* (Rut)*, Philotheca spicata* (Rut) and *Diploaenea sp* (Rut) **(DIXON *et al.,* 1995; ROCHE *et al.,* 1997)**. Butenolide and SW were effective in promoting germination of *Dioscorea dregeana* **(KULKARNI *et al.,* 2007)***.*

Smoke was identified as an important germination cue in post-fire events **(BROWN, 1993; BALDWIN and MORSE, 1994)**. The highly active compound has been identified to be 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one from burned cellulose **(FLEMATTI *et al.,* 2004)** and plant-derived smoke **(VAN STADEN *et al.,* 2004)**. The remarkable influence of smoke on seed germination is widely known and utilized in various ways **(ROCHE *et al.,* 1997; BROWN and VAN STADEN, 1998; VAN STADEN *et al.,* 2000)**. The identification of 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one has allowed for further research into the application of smoke technology for a variety of horticultural and agricultural crops **(VAN STADEN *et al.,* 2006)**. According to the authors, the effect of SW and butenolide extends beyond germination stimulation as it can also act to enhance seedling vigour and rapid plant growth. South African fynbos and Western Australian bush land have numerous species sensitive to smoke **(KEELEY and FOTHERINGHAM, 2000)** as half to two-thirds of the species tested gave a positive

response **(BROWN, 1993; DIXON *et al.,* 1995; ROCHE *et al.,* 1997; READ and BELLAIRS, 1999; TIEU *et al.,* 2001)**.

However, not all plant species respond positively to smoke-water treatment. A positive seed germination response to smoke-water and KAR_1 is common in plants which produce post-fire seedlings. The genus *Agathosma* (Rut) is closely related to *Coleonema.* Results of a germination study of *Agathosma apiculata* and *Agathosma stenopetala* did not support the prediction that seed germination of fynbos species is fire-stimulated **(PIERCE and MOLL, 1994)**.

2.1.6. Seed germination of Rutaceae plants

The Rutaceae family to which *Coleonema album* belongs is known to exhibit physiological dormancy. A review of seed dormancy classification for shrub species assumed that all Rutaceae had physiological dormancy **(OOI, 2007)**. In some cases of physiological dormancy, germination is stimulated by gibberellic acid (GA_3) **(BASKIN and BASKIN, 2004)**. Little is known about the role, timing and location of GA_3 activity in relation to environmental cues for germination, particularly for seeds from wild-sourced species. However, evidence suggests that smoke or smoke-water (SW) may increase the sensitivity of seeds to GA_3 and other hormones **(VAN STADEN *et al.,* 2000)**. Natural germination cues which were identified for seed germination of Rutaceae species include fire, heat and smoke **(PAYNTER and DIXON 1991; DIXON *et al.*, 1995; ROCHE *et al.,* 1997; AULD 2001)**. Seed germination of *Agathosma betulina* was improved by subjecting the seeds to heat at 80 °C for 20 min **(BLOMMAERT, 1972)**. Smoke stimulated the germination of *Geleznowia verrucosa, Philotheca spicata* and *Diploaenea sp* **(DIXON *et al.*, 1995; ROCHE *et al.*, 1997)**. Longevity of shrubby Rutaceae seeds is largely unknown **(AULD, 2001)**. The longevity of the South African fynbos Rutaceae seeds is suggested to be over 10 years **(LE MAITRE and MIDGLEY, 1992)**.

2.1.7. Is propagation through seed effective for Rutaceae plant species?

Seed germination of different plant species has been optimized in order to produce sufficient plant materials for human use. Mass propagation by seeds is an efficient and economic method of plant production **(HARTMAN *et al.,* 1975)**. However, seed germination of some plant species have proved difficult. Inherent seed dormancy, low seed viability, lack of germination cues, and production of immature embryos are among the challenges faced during plant propagation through seeds. All the above mentioned challenges are common to the Rutaceae family. Seed propagation in the Rutaceae is hampered by low seed numbers and poor viability **(MARTYN *et al.,* 2009)**. For example, *Geleznowia verrucosa* (Rut) is a popular bush-collected cut flower which fails to propagate reliably from seeds **(PAYNTER and DIXON 1990)**. Propagation of *Zanthoxylum piperitum* (Rut) by seeds is hampered by low viability, a low germination rate and delayed rooting of seedlings **(HWANG, 2005)**. The genus *Agathosma* (Rut) is closely related to *Coleonema* and some species of *Agathosma* have been reported to exhibit physiological dormancy, among them are *Agathosma apiculata*, *A. stenopetala* **(PIERCE and MOLL, 1994)** and *A. betulina* **(BLOMMAERT, 1972)**. Also other Rutaceae members such as: *Zanthoxylum mayanum* **(GONZALEZ, 1991)**, *Z. belizense*, *Z. setulosum*, **(GARWOOD, 1983)**, *Z. limonella*, *Aegle marmelos* **(BENIWAL and SINGH, 1989)** and *Spiranthera odoratissima* **(RIZZINI, 1965)** exhibit physiological dormancy. Seed viability in Rutaceae plants varies from one genus to the other and even among species of the same genus **(AULD, 2001)**. Patterns of low seed viability have been found within some genera while other genera showed favourable viability percentages. For example, low seed viability has been found in some *Boronia* species **(BELL *et al.,* 1993)** while high levels of seed viability was found in a *Diploaenea* sp and *Geleznowia verrucosa* **(ROCHE *et al.,* 1997)**. Seeds of the Rutaceae have a high level of dormancy immediately after release from the parent plant **(AULD, 2001)**.

Vegetative propagation has been used as an alternative method to produce young plants from adult plants. Unfortunately, some plant species are difficult to propagate vegetatively due to their inability to root successfully. According to **HWANG (2005)**, conventional propagation of *Zanthoxylum piperitum* through seeds, stem and root cuttings is too slow to provide adequate plant numbers to meet demands. Plant

tissue culture has served as a potential alternative for mass production of plants which are difficult to propagate from seeds and cuttings. Globally, it has contributed significantly towards the production of high quality plant materials.

2.2. Plant Tissue Culture

Plant tissue culture also known as *in vitro* propagation refers to the growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environments. The concept of tissue culture, which embodies the theory of totipotency was first postulated by Gottlieb Haberlandt in 1902 **(KRIKORIAN and BERQUAM, 1969)**. The theory states that given the right conditions, individual cells are capable of growing into whole plants.

2.2.1. Advantages of plant tissue culture

Plant tissue culture techniques offer various advantages over other methods of plant propagation which include:

i. The production of exact copies of plants that produce particularly good flowers, fruits or have other desirable traits within a short period of time. *In vitro* flowering is advantageous for species with a long juvenile period such as geophytes. Studies have shown that *in vitro* conditions significantly shortened the juvenile period in geophytes **(ZIV and NAOR, 2006)**;

ii. The regeneration of whole plants from plant cells that have been genetically modified;

iii. To clean plants of viral and other infections and to quickly multiply these plants as 'Cleaned Stock' for agricultural and horticultural purposes. For example, tissue culture techniques have been used for the production of high quality and pathogen-free potato (*Solanum tuberosum*) seed tubers **(CHIIPANTHENGA *et al.*, 2012)**;

iv. The production of plants in the absence of seeds or from seeds that otherwise have very low chances of germination and growth. For example, *Dierema*

erectum is a rare ornamental geophyte and its conventional propagation is hampered by seed parasitism. A micropropagation protocol of this plant was developed **(KOETLE *et al.,* 2010)** to serve as an alternative means of propagation of this rare plant;

v. Conservation of plants through cryopreservation. **FAY (1994)** reported that prolonged maintenance of *in vitro* material provides an effective system for establishing both active and base germplasm collections particularly for the conservation and maintenance of those plant species that cannot be stored as true seed and are amenable to micropropagation technology. Tissue culture techniques have served as a tool for the conservation and multiplication of medicinal plants **(BHOJWANI, 1980; WAWROSCH *et al.,* 2001; MARTIN, 2002, 2003; JOSHI and DHAR, 2003)**. The use of *in vitro* culture, cryopreservation, and molecular markers offer valuable alternatives for plant diversity studies, management of genetic resources and ultimately conservation **(PAUNESCU, 2009)**; and

vi. *In vitro* metabolite production in medicinal plants. Most valuable phytochemicals are products of plant secondary metabolism **(TRIPATHI and TRIPATHI, 2003)**. Cell lines capable of producing high yields of secondary compounds in cell suspension cultures have been successfully established **(ZENK, 1978)**. The production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants. Solasodine was produced from calli of *Solanum eleagnifolium*, and pyrrolizidine alkaloids from cultures of *Senecio* sp **(NIGRA *et al.,* 1987, TOPPEL *et al.,* 1987)**. Cephaelin and emetine were isolated from callus cultures of *Cephaelis ipecacuanha* **(JHA *et al.,* 1988)**, quinolone alkaloids in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana* **(SCRAGG, 1992)**. Different media modifications have been used to strategically improve secondary metabolite production in suspension cultures of different plant species **(ROBINS, 1994)**.

The success of the technique depends mainly on the culture environment (i.e. culture media components, temperature and light) and choice of explants. More than fifty different devised media formulations have been used for the *in*

vitro culture of tissues of various plant species **(GAMBORG *et al.*, 1976; HUANG and MURASHIGE, 1977)**. However, the most commonly used formulation was described by **MURASHIGE and SKOOG (1962)**. A nutrient medium basically consists of all the essential elements, vitamins, a carbon source, organic supplements and plant growth regulators.

1. Essential elements or mineral ions are supplied as a complex mixture of salts. They are categorized as: micro-elements and macro-elements.

   Macro-elements used in plant tissue culture include: nitrogen, magnesium, calcium, sulphur, phosphorus and potassium. These elements are required in relatively large amounts for plant growth and development **(GEORGE *et al.*, 2008)**.

   Micro-elements used in plant tissue culture include: manganese, cobalt, copper, boron, molybdenum, iron, zinc and iodine. They are required in trace amounts for plant growth and development **(GEORGE *et al.*, 2008)**.

2. Organic supplements supplies vitamins and amino acids. In early work, the requirements of tissue cultures for trace amounts of certain organic substances was fulfilled by the use of fruit juices, coconut milk, yeast or malt extracts and hydrolyzed casein as they contribute vitamins and amino acids to a culture media **(GEORGE *et al.*, 2008)**. According to the authors, the use of these supplements has declined as specific organic compounds has been defined and are today listed in catalogues as pure chemicals. The authors stated that the vitamins most frequently used in plant tissue culture media are thiamine, nicotinic acid and pyridoxine.

3. The carbon source is usually supplied in the form of sucrose. Sucrose is generally regarded as a standard carbohydrate in plant tissue culture **(EVANS *et al.*, 1981; DEBNATH, 2005)**. The type of gelling agent mostly used in tissue culture is agar **(KAÇAR *et al*, 2010)**. It is produced from seaweed and has been found to be ideal for routine applications. High clarity, stability and resistance to metabolism during culture are properties that make agar the gelling agent of choice **(JAIN and BABBAR, 2002)**. Other gelling agents used in plant tissue culture systems include agarose, phytagel and gelrite **(DEBERGH, 1983)**. Plant

may respond differently in culture as a result of the gelling agent used. Shoot regeneration and hyperhydricity levels are greatly affected by the type of gelling agent **(IVANOVA and VAN STADEN, 2011)**. Various plant species have shown as good results on gelrite as on agar **(BHOJWANI and RAZDAN, 1996)**. According to the authors, certain plants however show hyperhydricity on gelrite due to more freely available water. The use of gelrite resulted in a significantly lower multiplication of *Aloe polyphylla* and almost four times hyperhydricity compared to agar solidified medium **(IVANOVA and VAN STADEN, 2011)**.

4. Plant growth regulators are critical components of the culture media in that they control and determine the development of plant cells in culture. They coordinate plant development at all levels; from the cellular level to organs and the whole plant.

2.2.2. Plant growth regulators

Plant growth regulators are low molecular weight natural products that regulate plant physiological and developmental processes at micro-molar or lower concentrations **(SKOOG and MILLER, 1957; BAJGUZ and PIOTROWSKA, 2009)**. Cytokinins and auxins are the most important groups of plant growth regulators that control plant growth and development in plant tissue culture systems.

Cytokinins promote cell division and morphogenesis in tissue culture. It also stimulates the release of apical dominance, and leaf expansion resulting from cell enlargement. Cytokinins promote the conversion of etioplasts into chloroplasts via stimulation of chlorophyll synthesis **(MAUSETH, 1991; RAVEN *et al.,* 1992; SALISBURY and ROSS, 1992; DAVIES, 1995)**. Other roles cytokinins play in plants are induction of cytokinesis, retardation of senescence **(GAN and AMASINO, 1995; KIM *et al*., 2006)**, phyllotaxis **(GIULINI *et al.,* 2004)** and reproductive competence **(ASHIKARI *et al.,* 2005)**. In intact plants, cytokinins regulate apical dominance and lateral shoot initiation **(SRIVASTAVA, 2002)**. Cytokinins are required for root formation and vascular morphogenesis in the roots **(MALÁ *et al.,* 2009)**.

Cytokinins are involved in the regulation of various genes encoding transporters for nitrate, ammonium, sulphate, phosphate and iron **(SAKAKIBARA, 2006; SEGUELA *et al.*, 2008)**. This metabolic activity is known to mediate primary uptake and proper allocation of essential nutrients. Cytokinins play an important role in efficient nutrient acquisition and usage **(KUDO *et al.*, 2010)**. Zeatin, N^6-Benzyladenine and 2-isopentenyladenine are naturally occurring cytokinins while kinetin is a synthetic analogue.

The differences in cytokinins are related to their structural features such as the side chain attached to the adenine group (FIG 2.1), their conjugation with sugars and phosphorylation **(GALUSZKA *et al.*, 2008)**. These structural variations are responsible for observable biological activities of cytokinins **(SAKAKIBARA, 2006)**. The most frequently used cytokinin in plant tissue culture is N^6-Benzyladenine (BA) as it is cheap and effective compared to the other cytokinins. However, various shortcomings have been attributed to the use of N^6-Benzyladenine in micropropagation protocols. It is reported to cause hyperhydricity in many species **(LESHEM and SACHS, 1985; LESHEM *et al.*, 1988; TERAMOTO *et al.*, 1993)**.

The search for a better alternative to N^6-Benzyladenine led to the discovery of a new group of aromatic cytokinins from poplar leaves **(STRNAD, 1997)**. According to **STRNAD (1997)**, the cytokinins were named Topolins which was derived from Topol the Czech word for poplar. Since the discovery of topolins as naturally occurring aromatic cytokinins, they have emerged as genuine alternatives to the long serving cytokinins (N^6-Benzyladenine, zeatin and kinetin) **(AREMU *et al.*, 2012)**.

Globally, the past 15 years has witnessed a surge in the use of topolins and their derivatives in research laboratories **(AREMU *et al.*, 2012)**. According to the authors, several positive reports on shoot multiplication rate, alleviation of physiological disorders, better acclimatization and rooting have made topolins popular amongst plant tissue culturists. The superiority of certain topolins over some commonly used cytokinins in micropropagation of different plant species have been demonstrated in various tissue culture protocols. For example, *meta–topolin* influenced a high shoot formation frequency in sugar beet compared to N^6-Benzyladenine and zeatin **(KUBALÁKOVÁ and STRNAD, 1992)**. Improved rooting in *Spathiphylum floribundum*

by *meta*–topolin compared to N^6-Benzyladenine has been reported **(WERBROUCK *et al.,* 1996)**. The use of *meta*–topolin improved shoot multiplication of *Curcuma longa* compared to N^6-Benzyladenine, kinetin and zeatin **(SALVI *et al.,* 2002)**. Topolins improved; survival rate of *in vitro* grown potatoes **(BAROJA-FERNANDEZ *et al.,* 2002),** shoot multiplication of *Sisyrinchium laxum* **(ASCOUGH *et al.,* 2011)** and *Pinus sylvestris* **(DE DIEGO *et al.,* 2010)** and fruit weight of *Prunus avium* **(ZHANG and WHITING, 2011)**.
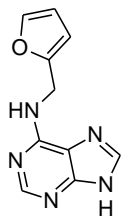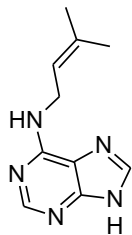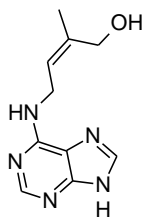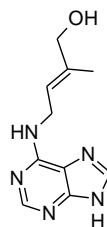
6-Furfurylaminopurine adenine N^6-(Δ^2-isopentenyl)   *Trans*-zeatin   *Cis*- zeatin   Thidiazuron
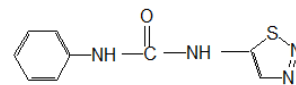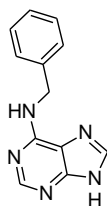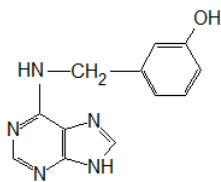
N^6-Benzyladenine   *meta*-Topolin   *meta*-Methoxytopolin riboside   *meta*-Topolin riboside   *meta*-Methoxytopolin
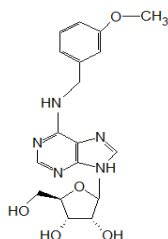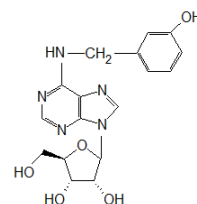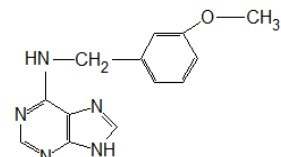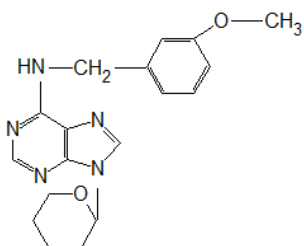
meta-Methoxytopolin tetrahydropyran-2-yl

FIGURE 2.1: Structures of cytokinins used in plant tissue culture.

Auxins positively influence cell enlargement, bud formation, root initiation and growth. Indole-3-acetic acid (IAA) is regarded as the most common naturally occurring auxin in plants **(MUDAY and DELONG, 2001)**. However, IAA is unstable to heat unlike the synthetic forms such as 2,4-dichlorophenoxyacetic acid (2,4-D), Indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) (FIG 2.2). Auxins promote the production of other growth regulators and in conjunction with cytokinins, control the growth of stems, roots, and fruits **(DAPHNE and McMANUS, 2005)**. Auxin regulates the development of primary and lateral roots **(MALAMY and BENFEY, 1997; SABATINI *et al.,* 1999; CASIMIRO *et al.,* 2001; TAIZ and ZEIGER, 2002; BENKOVÁ *et al.,* 2003; RAVEN *et al.,* 2005; TEALE *et al.,* 2005; WOODWARD and BARTEL, 2005)** and root vascular differentiation **(SACHS, 1981; ALONI, 2004)**. Auxin promotes the formation of adventitious roots **(FALASCA *et al.,* 2004; SORIN *et al.,* 2005)**. They stimulate cambium, a subtype of meristem cells to divide and in stems cause secondary xylem to differentiate. Leaf abscission is initiated by the growing point of a plant ceasing to produce auxins. Auxins in seeds regulate specific protein synthesis **(WALZ *et al.,* 2002)**, as they develop within the flower after pollination, causing the flower to develop a fruit which contains the developing seeds.
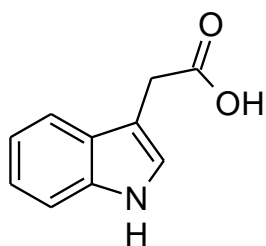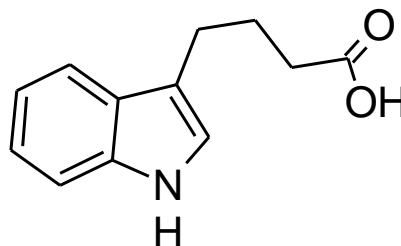
Indole-3-acetic acid   Indole-3-butyric acid

2, 4-Dichloropheneoxyacetic acid   α-Naphthalene acetic acid

FIGURE 2.2: Structures of commonly used auxins.

Gibberellins are involved in various developmental processes in plants. Its actions in plants include: stimulation of seed germination, leaf expansion, flower induction and fruit development **(ROSS *et al.,* 1997; SUN and GUBLER, 2004)**. Gibberellins do not play a significant role in *in vitro* development regulation. However, they have been used to enhance shoot elongation before rooting or to stimulate conversion of buds into shoots **(GABA, 2005)**. Gibberellins may interfere with bud initiation, reduce root formation and interfere with embryogenesis **(TRIGIANO and GRAY, 2011)**.

Ethylene controls fruit ripening and is not commonly used in plant tissue culture as it can inhibit growth and development of culture systems if it builds up in high amounts. Ethylene is involved in senescence **(LIM *et al.*, 2007),** abscission **(LEWIS *et al.*, 2006),** and root formation **(SWARUP *et al.*, 2007; NEGI *et al.*, 2008)**.

Abscisic acid is used to promote a distinct developmental pathway such as somatic embryogenesis as it inhibits cell division. Abscisic acid regulates many processes during the plant life cycle. This includes key events during seed formation, such as: the deposition of storage reserves, prevention of precocious germination, the acquisition of desiccation tolerance and the induction of primary dormancy **(KERMODE, 2005)**.

Auxin and cytokinin are the most commonly used plant regulators in plant tissue culture techniques and are usually used together. Root and shoot initiations are closely regulated by the concentrations of auxins and cytokinins in the medium **(SKOOG and MILLER, 1957; BAJAJ *et al.*, 1988; ROUT and DAS, 1997)**. An auxin to cytokinin ratio of 10 yield rapid growth of undifferentiated callus, a ratio of 100 favours root

development and a ratio of 4 favours the development of shoots **(MURASHIGE, 1980)**. Many developmental processes in plants such as cell growth, cell division and differentiation as well as organogenesis in tissue and organ cultures are controlled by an interaction between cytokinins and auxins **(GASPAR *et a*l., 1996)**. An appropriate balance between the two is required to initiate plant growth **(GASPAR *et al.,* 1996)**. Different ratios are used in callus culture for the production of callus depending on the type of explants and plant species.

Light conditions are important factors in micropropagation systems. They influence micropropagation through three different parameters: photoperiod, intensity and spectral quality **(MURASHIGE, 1974; ECONOMOU and READ, 1987)**. These parameters act to influence the levels of endogenous plant growth regulators which may result in various morphological responses in different plant species **(MACHÁČKOVA *et al.,* 1992; TAPINGKAE and TAJI, 2000)**. They can improve plant quality and growth rate **(ECONOMOU and READ, 1987)**. The developmental processes in plants that are influenced by photoperiod include: rooting and stem elongation **(RAMANAYAKE *et al.,* 2006)**. The wavelength values required for tissue culture plantlet range from 400 to 700 nm **(CHEN, 2005)**. The most favourable growth condition for most plants is a 16/8 h light/dark cycle **(MORINI *et al.,* 1991; READ, 2007)**. It is necessary to determine the minimum photoperiod required for satisfactory plant growth *in vitro* **(MORINI *et al.,* 1991)** as a small increase in the photoperiod can substantially increase the cost of *in vitro* propagation. According to **LEE (1994)**, increased light level *in vitro* may positively influence the quality of some plantlets and negatively affect the growth and quality of other species. Full light intensity was suggested to be a critical condition for shoots production, while darkness in the root zone was essential for positive results with *Cymbidium* **(WECKMEISTERR, 1971)**. High light intensities have been reported to be satisfactory for potato **(PENNAZIO and RADOLFI, 1973)** and Asparagus **(HASEGAWA *et al.,* 1987)** while low light intensities **(WAINWRIGHT and FLEGMAN, 1984; ECONOMOU and READ, 1987)** have also been demonstrated to be necessary for several species.

2.2.3. Stages involved in micropropagation

Successful *in vitro* regeneration of plants depends on the completion of the different sequential stages involved. The four stages (I-IV) proposed by **MURASHIGE (1978)** are widely accepted. **DEBERGH and MAENE (1981)** suggested an additional stage 0. These stages have been used globally in the development of *in vitro* propagation protocols for various plant species.

2.2.3.1. Stage 0: Selection of mother plant

This stage involves the selection of healthy mother plants capable of being uniformly initiated into culture **(DEBERGH and MAENE, 1981)**. A healthy mother-plant free of any symptoms of disease or pathogenic infection must be selected **(CONSTABEL and SHYLUK, 1994)**. The impact of stage 0 is beyond the sanitary situation of the explants as it also influences the rate of survival **(DEBERGH and MAENE, 1981)**. The nutritional state of the stock plants from which explants are taken can influence the success of *in vitro* plant regeneration **(DEBERGH and MAENE, 1985)**. Growing of stock plants in a controlled condition prior to *in vitro* culture will yield healthier explants **(DEBERGH and MAENE, 1981)**. According to **DEBERGH and MAENE, (1985)**, a clean starting material will need a weaker sterilization which will result in less problems of browning of the tissues.

2.2.3.2. Stage I: Establishment of an aseptic culture

Young plant parts are taken from the mother plant as they are totipotent. Explants are put through surface decontamination procedures in order to be able to raise healthy plants *in vitro*. This process of surface decontamination of explants taken from the mother plant is a necessary prerequisite prior to *in vitro* culture **(TENG *et al.*, 2002)**. Chemical substances commonly used for decontamination include: 70% ethanol, mercuric chloride and sodium hypochlorite.

2.2.3.3. Stage II: Propagation of plant materials/explants

In this stage different methods of *in vitro* propagation are used. They are:
Propagation from axillary buds/shoots involves the use of shoot-tips or nodes as explants. Formation of adventitious shoots or embryos can be achieved directly by the use of tissues (or organs) or indirectly by the formation of callus. Often, they develop from sites where meristems do not exist **(RAZDAN, 2003)**. However, *in vitro* production of plants from axillary buds has proved to be the most commonly used and reliable method of micropropagation. Shoot proliferation from the axillary shoot is considerably slower compared to other multiplication methods but it allows annual multiplication rates that surpass conventional propagation by cuttings **(MURASHIGE, 1978)**. Adventitious organogenesis is the most preferable method of multiplication as it enables for a rapid increase of propagula **(DEBERGH and MAENE, 1981)**.

2.2.3.4. Stage III: Preparation for growth in natural environment

Shoots regenerated *in vitro* cannot survive without a proper and functioning root system when grown in the soil. Therefore, *in vitro* root formation is an important stage subsequent to *in vitro* shoot production **(MURASHIGE, 1978)**. Rooting of shoots *in vitro* is the most labour-intensive stage of micropropagation as it is the only step in which single shoots must be handled **(DEBERGH and MAENE, 1981)**. The most widely used auxin for rooting in commercial micropropagation is indole-3-butyric acid **(DE KLERK *et al.*, 1999)**.

2.2.3.5. Stage IV: Acclimatization

During this stage, the plants are transferred from the protective, sterile, humid climate to a transitional phase to be able to survive the dry, harsh weather of the field. Root production *in vivo* was proposed as an effective alternative by **DEBERGH and MAENE (1981)** so as to overcome shortcomings associated with *in vitro* rooting of tissue cultured shoots. Induction of a good functioning root system *in vitro* is very difficult **(DEBERGH and MAENE, 1981)**. Rooting mixtures for *in vitro* regenerated plants include: peat, vermiculite, bark, pumice, perlite, soil, sand, rockwool or their

mixtures with or without supplementation with fertilizer or lime **(DEBERGH and MAENE, 1981; TORRES, 1989)**. There is a need to acclimatize *in vitro* raised plants in a condition with slow decrease in humidity to prevent dehydration **(GEORGE and DEBERGH, 2008)** and this make mist house an important facility for the acclimatization process. Greenhouses also serve as a good growth facility for plant acclimatization as the temperature and humidity can be controlled throughout the year.

2.2.4. Tissue culture of Rutaceae species

The available literatures do not list a report on the *in vitro* propagation of *Coleonema album*. However, successful micropropagation of Rutaceae species, the family to which *Coleonema album* belongs has been reported (**Table 2.1**).

TABLE 2.1: List of some Rutaceae species propagated through plant tissue culture

Plant name	Explant source	PGR(s) for shoot initiation	Reference
Aegle marmelos	Cotyledons and shoot-tips	BA+NAA	**DAS *et al.*, 2008**
	Nodal explants	BA+IAA	**GUPTA *et al.,* 2008**
	Nodal segments	BA	**PUHAN and RATH, 2012**
	Nodal stem segment	BA	**WARRIER *et al.,* 2010**
Citrus halimii	Hypocotyls segments	BA	**NORMAH *et al.*, 1997**
Citrus limon	Nodal shoot segments	BA	**RATHORE *et al.*, 2007**
Citrus reticulata	Shoot-tips and nodal segments	BA and Kinetin	**MUKHTAR *et al.*,2005**
Feronia limonia	Shoot-tips and nodal segments	BA	**ISLAM *et al.*, 2010**
Murraya koenigii	Immature seeds	BA	**RANI *et al.*, 2011**
Naringi crenulata	Nodal segments	BA and Kinetin	**SINGH *et al.*, 2011**
Phebalium equestre	Shoot-tips and nodal segments	BA	**JUSAITIS, 1991**
Ruta graveolens	Leaf derived callus	BA+NAA	**AHMAD *et al.*, 2010**
	Nodal segment	BA+IAA	**BOHIDAR *et al.,* 2008**
	Nodal segments	BA	**DE CASTRO and DE BARROS, 1999**
	Nodal segments	BA+NAA	**FAISAL *et al.*, 2005**
Zanthoxylum piperitum	Shoot-tips	BA	**HWANG, 2005**

After scrutinizing available literature, it is evident that there is no report of seed and *in vitro* propagation requirements of any member of the genus *Coleonema*. Germination cues of Rutaceae family plants were used to optimize germination of *Coleonema album* seeds. Available literature on the micropropagation protocols of members of the Rutaceae family reflects that the use of BA is effective for *in vitro* propagation. However, the use of BA in plant tissue culture techniques has been generally reported to be associated with various shortcomings. The topolins have shown satisfactory results for *in vitro* micropropagation of various plant species. In this study the effect of the long serving cytokinins such as BA, kinetin, TDZ was investigated. The topolins have shown promising activities in various *in vitro* propagation protocols. Hence, the effect of topolins on *in vitro* regeneration of *Coleonema album* was compared to the long serving cytokinins.

CHAPTER 3. OPTIMIZING *COLEONEMA ALBUM* SEED GERMINATION

3.1. Introduction

Many rural South Africans depend on medicinal plants for their primary health-care **(KENDLER *et al.,* 1992; CUNNINGHAM, 1994; MANDER, 1998)**. There is a need for domestication and commercial cultivation of many species utilized in traditional medicine in order to meet their future demands **(VAN STADEN, 1999; JÄGER and VAN STADEN, 2000)**. The demand for medicinal plants has increased steadily since the start of the 20th century **(MANDER, 1997)**. Plant propagation through seeds has been an effective means for the provision of plant materials since the existence of man. The genetic diversity found in seeds provides the genes from which plant life covers most of the earth surface in all of its environmental variations **(HARTMAN *et al.,* 1975)**.

Botanically, a seed is a matured ovule containing an embryo that is usually the result of sexual fertilization **(ESAU, 1977; FAHN, 1982)**. According to **HARTMAN *et al.,* (1975),** every seed consists of a protective outer covering, storage tissue and an embryo. Seeds and fruits of different species vary greatly in appearance, size, shape, and location and structure of the embryo in relation to storage tissues. These points are not only useful for taxonomy and identification but are also key determinants for germination requirements of plant species. *Coleonema album* seeds have a brownish black colour. Seed size range between 2.6 mm to 3.4 mm in length and 1.4 mm to 1.7 mm in width. Seed micropyle diameter ranges between 0.28 mm to 0.40 mm. The seed coat is shiny and lustrous on the outer surface (FIG.3.1).
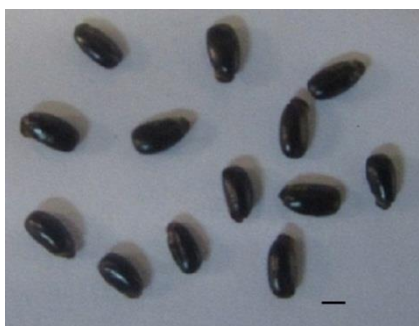
FIGURE 3.1: *Coleonema album* seeds. Scale bar represents 1 mm.

Propagation by seeds is the major method by which plants reproduce in nature and crops are cultivated **(HARTMAN *et al.,* 1975)**. However, raising plants from seeds can be erratic if specific knowledge of seed germination requirements is inadequate or absent. These requirements vary from one species to another, which includes: moisture content, temperature, light/darkness, scarification, stratification (cold or warm), sowing depth, salinity and pH. In South Africa, the successful cultivation of medicinal plants is determined to a large extent by germinability of the seeds **(KULKARNI *et al.,* 2005)**.

The germination requirement of seeds of this medicinal and ornamental plant is not well defined. It has been reported that problems are likely to arise in trying to grow species of the Rutaceae family from seeds as seed viability is variable and germination cues are poorly understood **(ROCHE *et al.,* 1997; AULD, 2001; FLOYD, 2008)**. Physiological dormancy is the most common type of dormancy in temperate species of Rutaceae **(BASKIN and BASKIN, 1998)**.

The major environmental factor causing changes in dormancy states is temperature, but other factors, including darkness, light, gases, chemicals and water, may be important **(BASKIN and BASKIN, 1978)**. Seed germination is a complex process involving many individual reactions and phases, each of which is affected by temperature **(COPELAND, 1976)**. The author stated that the effect of temperature on germination can be expressed in terms of cardinal temperatures- that is, minimum, optimum and maximum temperatures at which germination will occur. The optimum temperature is the one which gives the greatest percentage of germination within the shortest period of time.

The role of temperature and photoperiod on seed germination of most plant species is interwoven. For example, the highest percentage of *Eucomis autumnalis* seed germination was achieved when seeds were subjected to a constant darkness condition at 25 ± 2 $^{\circ}$C **(KULKARNI *et al.,* 2006)**. The highest percentage seed germination of *Drimia robusta* was recorded at a constant temperature of 20 $^{\circ}$C (87%) and alternating temperature of 25/20 $^{\circ}$C (90%). The authors recorded 93% germination under constant light and 80% in the dark.

In a germination study of valuable South African medicinal plants, seed germination of the majority of the species investigated was significantly promoted by exposure to continuous or alternating light compared to continuous dark. In the study, *Tulbaghia alliacea* and *Dianthus basuticus* germinated equally well in light and dark while *Urginea capitata* responded significantly to the continuous light treatment **(KULKARNI *et al.*, 2005)**.

However, the temperature and photoperiod requirement for seed germination of plant species in a controlled environment might be related to their geographical distribution. The relationship between *Coleonema album* seed germination requirements and its geographical distribution is not known.

3.2. Objectives

The objectives of this study were to:

i.   Improve germination of *Coleonema album* seeds by establishing optimal temperature and light requirements for germination;

ii. Investigate the influence of smoke-water, smoke-isolated KAR_1 and plant growth regulators on seed germination;

iii. Study the temperature requirements for seedling growth and survival.

3.3. Material and methods

3.3.1. Seed collection

Seeds of *Coleonema album* used in this study were purchased from the South African National Biodiversity Institute in Kirstenbosch. The seeds were harvested during spring 2011. The seeds were stored in airtight pill vials placed in a freezer set at -18°C.

3.3.2. Seed viability

Viability of the seeds was tested using tetrazoliumchloride (TTC) solution (1%) **(ISTA, 1999)**. Seeds were cut into two longitudinal halves and soaked in TTC in pill vials. Four replicate of twenty-five seeds each was used. The pill vials were wrapped

with aluminium foil and kept in the dark for two weeks at 25 ± 2 °C. A red/pink coloration of the embryos indicated viability of the embryos.

3.3.3. Moisture content

Two methods were used to determine the moisture content of *Coleonema album* seeds.

i.   Twenty-five seeds in four replicates were weighed and dried in an oven set at 110°C. Seeds were weighed daily until there was no further weight loss.

ii. Twenty-five seeds in four replicates were weighed and ground. They were subsequently placed in an oven set at 110 °C. Seeds were weighed until there was no further weight loss.

The moisture content was expressed as percentages on the basis of the original fresh weight using the equation below **(BEWLEY and BLACK, 1982)**:

Moisture content (%) = $\left(\dfrac{\text{Fresh weight of seed} - \text{Dry weight of seed}}{\text{Fresh weight of seed}}\right)$ x 100

3.3.4. Imbibition studies

Four replicates of twenty-five seeds each were placed on two layers of filter paper (Whatman number 1) moistened with 3 ml of distilled water (once in two days) in Petri-dishes (65 mm diameter) and allowed to take up water at room temperature (25 ± 2 °C). The seeds were weighed at 24 h intervals until there was no increase in seed mass. At each of the intervals, seeds were blotted dry with paper towel, weighed and then replaced on the wet filter papers.

3.3.5. Germination studies

Seeds of *Coleonema album* were decontaminated by soaking in 70% ethanol for two minutes followed by 0.2% mercury chloride ($HgCl_2$) for 15 min and then rinsed several times with distilled water. Seeds were subsequently placed on two layers of filter paper moistened with 3 ml distilled water or test solutions in disposable Petri-dishes (65 mm diameter). The filter papers were kept wet by adding water or the test solutions when necessary. Four replicates of twenty-five seeds each were used for each

experiment. Experiments were terminated after 90 days unless otherwise stated. Mean germination time (MGT) was determined using the formula:

$$MGT = \Sigma \frac{(n \times d)}{N}$$

Where n = number of seeds germinated on each day, d = number of days from the beginning of the test and N = total number of seeds germinated at the termination of the experiments **(ELLIS and ROBERTS, 1981)**. The data obtained were arcsine transformed before analysis for homogeneity.

3.3.5.1. Effect of temperature

The influence of different temperature regimes on seed germination was determined by incubating the seeds at constant temperatures (10, 15, 20, 25, 30, 35 °C) and an alternating temperature of 30/15 °C. After 90 days, seeds incubated at temperatures 20, 25, 30, 35 and 30/15 °C were transferred to a conviron set at 15 °C.

3.3.5.2. Effect of photoperiod

The effect of light on germination was evaluated under cool white fluorescent lamps (photosynthetic photon flux density of 40.5 µmol m^{-2}s^{-1}) alternating light (16 h photoperiod) and constant dark in which seeds in Petri-dishes (65mm diameter) were kept in light-proof boxes and germination was recorded under a green safe light (0.3 µmol m^{-2} s^{-1}) at 25 ± 2 °C daily.

3.3.5.3. Effect of cold and warm stratification

Four replicates of twenty-five seeds each were wrapped in-between wet paper towels. The wet paper towel were wrapped with aluminium foil and kept inside air-tight plastic bags. Four of the plastic bags were kept inside an incubator set at 5 °C for the cold stratification experiments. For the warm stratification experiment, the plastic bags were kept in an incubator set at 30 °C. The seeds were observed under a safe green light every 3 days during which the paper towels were moistened with distilled water.

3.3.5.4. Effect of plant growth regulators

Test solutions used include: kinetin, BA, IBA and NAA. Four concentrations (10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M) of each test solution were used. The experiment was carried out under alternating light (16h light/8h dark) and constant dark conditions at 15 °C.

3.3.5.5. Effect of smoke solutions

Seeds were incubated with smoke water (1:250, 1:500, 1:750 and 1:1000 v/v) and KAR_1 (10^{-6}, 10^{-7} and 10^{-8} M) isolated from smoke **(VAN STADEN *et al.,* 2004)**. The experiment was carried out under alternating light and constant dark conditions at 15 °C. The smoke-water was prepared by the methods outlined by **BAXTER *et al* (1994)**.

3.3.5.6. Effect of pH and salt solutions

Solutions of pH 4, 5, 6, 7, 8, 9 and 10 were prepared using potassium hydroxide and hydrochloric acid solutions. Seeds were incubated with different pH solutions. The experiment was carried out under constant dark conditions at 15 °C. Twenty-five seeds in four replicates were used for each of the treatments.

Seeds were incubated with sodium chloride (NaCl) and ammonium nitrate (NH_4NO_3) at 15 °C for two weeks. Afterwards, distilled water was used to wet the seeds for the rest of the germination period. Three concentrations (10^{-1}, 10^{-2} and 10^{-3} M) of each salt solution were used. The experiment was carried out under constant dark conditions at 15 °C. Twenty-five seeds in four replicates were used for each of the treatments.

3.4. Results and discussion

3.4.1. Seed viability, moisture content and water uptake

After TTC treatment, 90% of the seeds showed pink to red coloration which indicated that they were viable. After physiological maturity, the viability of seeds

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gradually declines. Their longevity depends on the environmental conditions to which they are exposed **(COPELAND, 1976)**. It was observed that the seeds (crushed) have high oil content. Thus, the high moisture content of the seeds (35%) may be due to the oil.

The *Coleonema album* seed coat is permeable, water content was highest on the sixth day after which rate of imbibition started to decrease (FIG 3.2). Seed germination is a complex chain of reactions which starts with moisture uptake. The uptake of water by seeds is an essential initial step towards germination. The total amount of water taken up during imbibition is generally quite small and may not exceed two or three times the dry weight of the seed **(BEWLEY and BLACK, 1994)**. According to the authors, the rate of water penetration into the seed is critical to the success of germination. The authors stated that if water uptake is too slow, then germination is reduced because seeds may deteriorate; if water uptake is too rapid, seeds may suffer excess imbibition damage. Imbibition differs greatly between seeds in relation to seed size and weight, structure, permeability, chemical composition, variation in temperatures and seed-water contact areas **(OBROUCHEVA and ANTIPOVA, 1997)**.
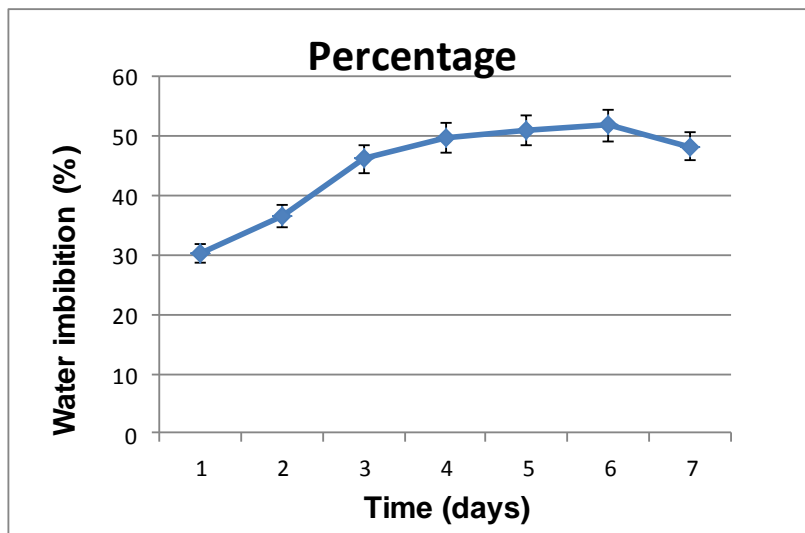
FIGURE 3.2: Imbibition rate of *Coleonema album* seeds at 25 ± 2 °C under a
16 h photoperiod.

3.4.2. Effect of temperature on germination of Coleonema album seeds

Coleonema album seeds germinated at low temperatures (10 and 15 °C) while high temperatures completely inhibited seed germination (FIG 3.3a). This is an indication that seeds of *C. album* will remain dormant at high temperatures. The highest germination percentage recorded (47.5%) was from 15 °C (constant darkness) with MGT of 36 days (as shown in FIG 3.3a). According to **COPELAND (1976)**, the optimum germination temperature for most seeds is between 15 and 30 °C while some species will germinate at temperatures approaching freezing point. The author emphasized that response of seeds to temperature depends on the species, variety, growing regions and duration of time from harvest. According to the author, seeds from temperate region require lower temperatures than those from tropical region. *Coleonema album* is a temperate species and it germinates during the winter period when soil temperatures are low. The confined distribution of this species in South Africa to certain areas of the country where the winter temperatures are low can be attributed to the fact that seeds can only germinate within a narrow range of temperature.

Coleonema album seeds incubated at high temperatures were transferred to a low temperature (15 °C) after 90 days of incubation. These temperature shifts showed favourable germination responses. Temperature shift from 20 °C to 15 °C was the most favourable for seed germination (as shown in FIG 3.3a) with 67.5% germination. These temperature shifts gave a higher germination percentage of *Coleonema album* seeds under alternating light than constant dark conditions. The result of this study shows that the light requirement for germination may be influenced by the temperatures the seeds are exposed to during germination. Similarly, **EVENARI, (1952)** reported that seeds of *Lactuca sativa* (cultivar- Grand Rapids) germinated above 80% in light at temperatures of 10 to 30 °C, whereas germination in darkness reached 45% only at temperatures of 10 to about 22 °C; it was near 0% at 30 °C. Seeds of *Bidens pilosa* **(FELIPPE, 1978)**, *Cynodon dactylon*, *Deschamsia caespitose* **(THOMPSON *et al.*, 1977),** *Nicotiana tabacum* **(TOOLE *et al.*, 1957)** required light to germinate at constant temperatures, but they germinate in light and in darkness at alternating temperatures.

The different phases involved in seed germination are affected by temperature **(COPELAND, 1976)** and the optimum temperature is the one which gives the greatest percentage of germination. According to the author, temperature responses may change throughout the germination period as each stage has its own cardinal temperature. *Coleonema album* seeds are ejected by a catapult mechanism in the field. This seed dispersal takes place early in the year when the soil temperatures are higher than what is required to break seed dormancy of this species. However, the high temperature may be involved in rapid seed imbibition and rupture of the seed coat in preparation for germination during the autumn. The temperature decreases gradually from high during December, January, February and March to a moderate temperature in April. Subsequently, seeds are exposed to temperatures ranging from moderate to cold during April and May then a low winter temperature in June and July. The exposure of seeds to high temperatures for 2 to 4 months during the summer period may have an influence on its response to germination at low temperatures afterwards. Several studies have shown that seeds of winter annuals must be exposed to high summer temperatures for several months to germinate in autumn **(ROBERTS and NEILSON, 1982; BASKIN and BASKIN, 1984a; STANDIFER and WILSON, 1988)**. Similarly,

seeds of *Hyacinthoides non-spripta* require several weeks of incubation at 26-31 °C followed by a germination phase at 11 °C **(BEWLEY and BLACK, 1994)**.

The crucial function of dormancy is to prevent germination when conditions are suitable for germination but the probability of survival and growth of the seedling is low **(FENNER and THOMPSON, 2005)**. Seeds of most obligate or facultative winter annuals mature in spring and/or early summer and are either dormant or conditionally dormant (**BASKIN and BASKIN, 1998)**. When seeds of winter annuals are in a state of conditional dormancy, shortly after dispersal, germination is characteristically restricted to a narrow range of low temperatures **(BASKIN and BASKIN, 1971b, 1972, 1978; PEMADASA and LOVELL, 1975)**. These dormant seeds will germinate only at low temperatures (5, 10, 15, 15/6, and 20/10 °C) which do not occur in the habitat in the late spring or summer **(BASKIN and BASKIN, 1983)**.

Temperature requirement for seed germination in a controlled condition determines the best sowing time of the year. Germination of *Coleonema album* seeds was totally inhibited at high temperatures as they remained dormant until they were exposed to a low temperature. This explains why seeds of this plant germinate in the field during the winter period. Similarly, **WASHITANI** and **MASUDA (1990)** reported that the temperatures at which seeds of 39 co-occurring species in Japanese grassland began to germinate, when subjected to gradually increasing temperatures in a standardized screening programme, was closely related to the observed timing of emergence in the field.

In this study, it was observed that a high temperature is detrimental to the growth and survival of *Coleonema album* young seedlings. A high percentage (70%) of seedlings did not survive when transferred to the field in September as the prevailing temperatures in Pietermaritzburg were high (results not shown). Seedling vigour and survival was enhanced when seedlings were raised in controlled incubators set at 25 ± 2 °C and 20 ± 2 °C. Thus, seedlings require shade or an environment with temperatures not exceeding 27 °C for the first two months after germination. However, once the seedlings are well established, they can survive at higher temperatures.
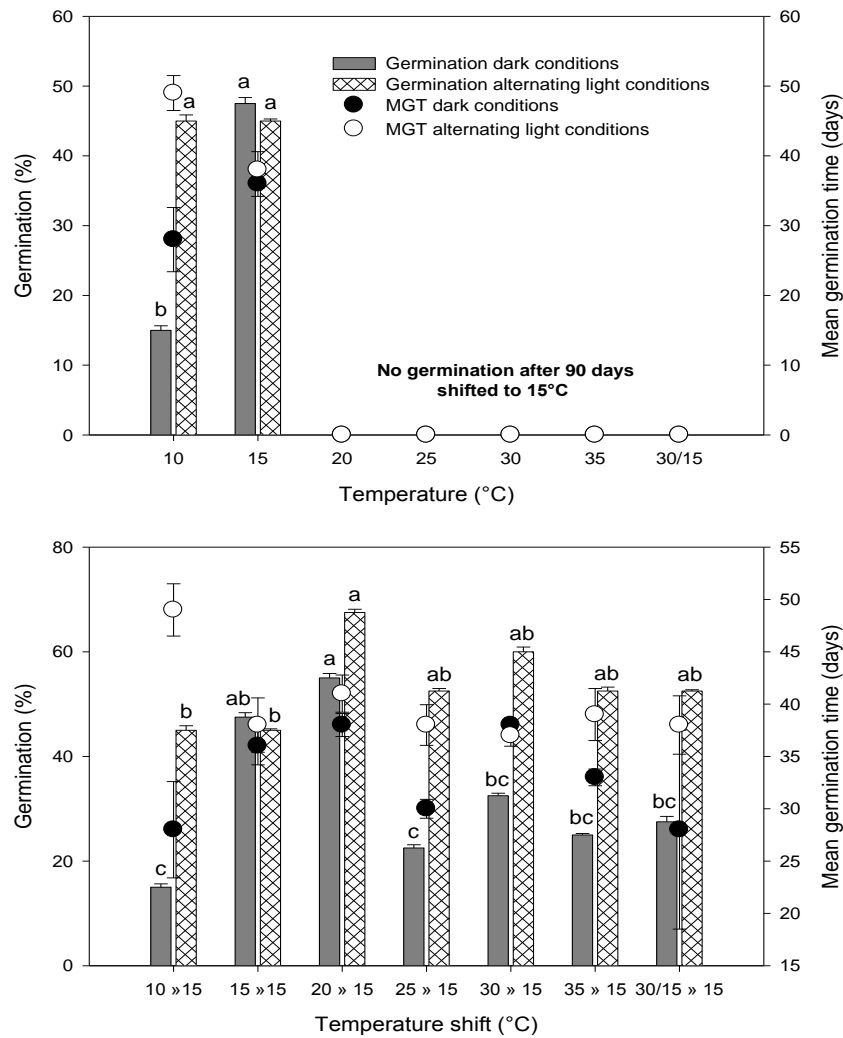
FIGURE 3.3a: Effects of photoperiod, different temperature regimes and temperature-shifts on germination of *Coleonema album* seeds under 16 h photoperiod and constant dark conditions at 15 °C. Bars (±SE) with different letters are significantly different (*p* < 0.05) according to Duncan's multiple range tests.

3.4.3. Effects of cold and warm stratification, plant growth regulators and smoke-water on seeds germination

Cold and warm stratification of seeds did not improve germination of seeds. All the plant growth regulators tested did not improve seed germination. In some plant species, the dormancy is broken by exposure of seeds to high temperatures and the dormancy is not completely broken or is not broken at all if seeds are cold stratified **(BASKIN and BASKIN, 1986)**. Smoke-water and KAR_1 did not improve seed germination percentage. Smoke-water and KAR_1 have been reported to stimulate seed germination of several plant species. However, not all plant species respond positively to smoke-water treatments. A positive seed germination response to smoke-water and KAR_1 is common in plants which produce post-fire seedlings. The genus *Agathosma* is closely related to the genus *Coleonema*. Results of a germination study with *Agathosma apiculata* and *A. stenopetala* did not support the prediction that germination of this fynbos species is fire-stimulated **(PIERCE and MOLL, 1994)**. *Boronia megastigma* produces post-fire seedlings **(VIGILANTE *et al.,* 1998)** but an attempt to germinate the seeds with direct smoke stimulation was unsuccessful **(DIXON *et al.,* 1995)**.

3.4.4. Effects of pH and salt solutions on germination of Coleonema album seeds

Seeds of *Coleonema album* incubated with pH 6 solution under constant darkness gave the highest germination percentage (52.5%). Germination percentages decreased in seeds incubated with higher and lower pH solutions (FIG 3.3b). This is an indication that *C. album* seeds have a wide range of hydrogen ion concentration levels at which seeds respond to germination with pH 6 as the optimum.

It has been reported that the germination of almost all species occurs readily between pH values of 4.0 and 7.6 **(JUSTICE and REECE, 1954)**. Seeds of many species germinate to high percentages over a wide range of pH values **(STUBBENDIEK, 1974; SINGH *et al.,* 1975; MABO *et al.,* 1988; RIVARD and WOODARD, 1989; ARTS and VAN DER HEIJDEN, 1990)**. However, some seeds germinate to high percentages only at a specific pH **(BASKIN and BASKIN, 1998)**. The optimum pH for the germination of *Calluna vulgaris* was 4.0 **(POEL, 1949)**, but it was

7.0 for seeds of *Bidens biternatum* **(AHLAWAT and DAGAR, 1980)** and 8.0 for those of *Tridax procumbens* **(RAMAKRISHNAN and JAIN, 1965)** and *Euphorbis thymifolia* **(RAMAKRISHNAN, 1965)**. Seeds of *Paulownia tomentosa* did not germinate at pH values of 1.5 to 3.5, but they germinated to 79 and 98% at pH 4.0 and 7.0 respectively **(TURNER *et al.*, 1988)**.

A concentration of 10^{-2} M sodium chloride and ammonium nitrate (both under constant darkness) gave highest percentage of seed germination of all three concentrations tested. Sodium chloride and ammonium nitrate (10^{-2} M) pre-treatments gave a 62.5% and 75% seed germination respectively (FIG 3.3b). Nitrate (NO_3^-) is one of the most ubiquitous and nutritionally important inorganic ions in soils. Along with the ammonium ion, it provides the main source of nitrogen to plants **(FENNER and THOMPSON, 2005)**. Nitrate gives a dormancy breaking response **(PONS, 1989)** and it has long been known to stimulate germination.

Coleonema album grows along the coastal areas of the Cape region. Ions present in the Cape coastal soils include sodium, chloride and nitrate. *Coleonema album* seeds are dispersed during the summer period during which evaporation rate is very high. Thus, salinity of the soil is very high during this period of the year. Subsequently, the autumn and winter rain reduces the soil salinity. On wet days, salt content of the soil may be diluted by rain **(FENNER and THOMPSON, 2005)**. The timing of seed germination in a saline habitat might be expected to coincide with periods when the soil water was diluted by rainfall and perhaps combined with a prevailing cooler temperature which would reduce evaporation **(FENNER and THOMPSON, 2005)**. Hence, the germination of *Coleonema album* seeds (dark condition) in the soil seed bank in its natural environment may be influenced by the interaction of these salts, the prevailing winter temperature conditions and the soil pH. Sodium chloride solution pre-treatment had a positive effect on the seed germination of the halophyte *Limonium emarginatum* **(REDONDO-GOMEZ *et al.,* 2008)**.
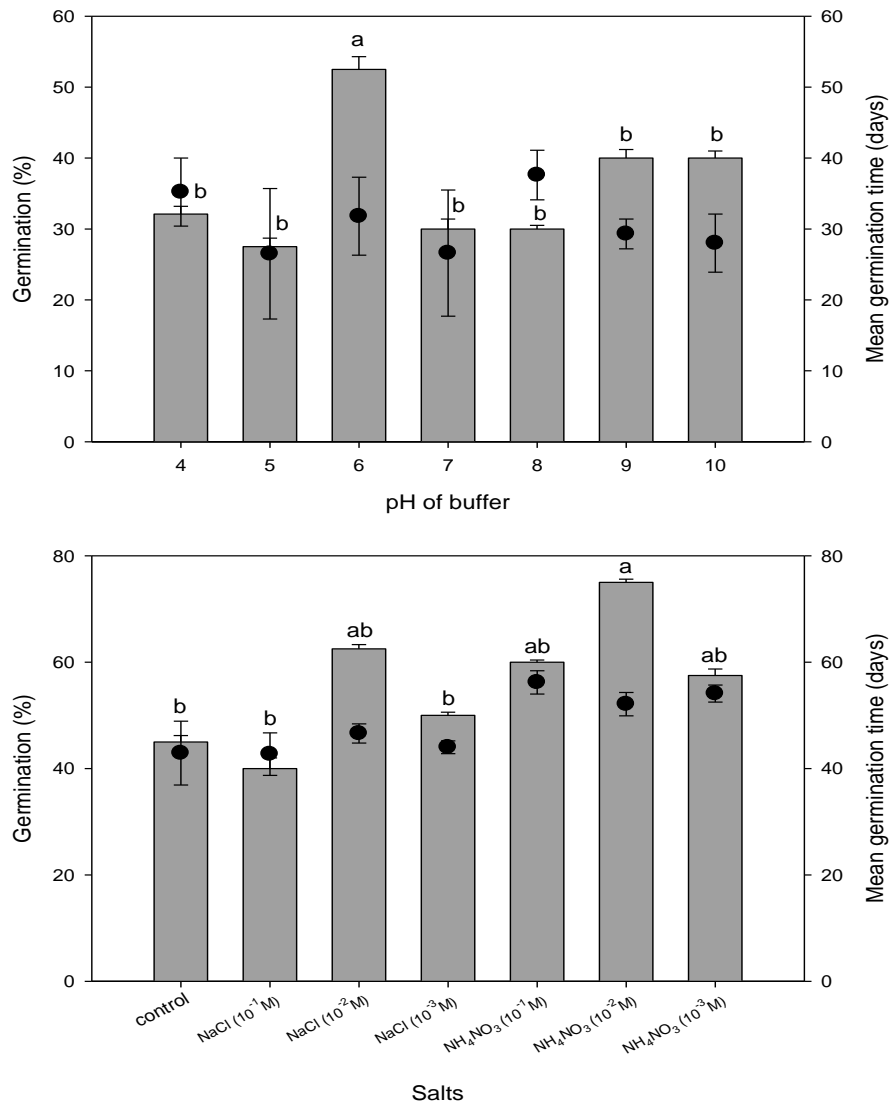
FIGURE 3.3b: Effects of pH levels and salt solutions on germination of *Coleonema album* seeds under a continuous darkness condition at 15 °C. Bars (±SE) with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test.

3.5. Conclusions

Coleonema album seeds exhibit physiological dormancy as germination of seeds is temperature-dependent. Seeds germinated within a narrow range of temperatures. Seeds require low temperatures for dormancy release. The most favourable temperature for germination of this species is 15 °C. Temperature shifts improved percentage germination of *C. album* seeds. Effect of photoperiod on seeds germination is temperature-dependent. A continuous dark condition favoured seed germination at 15°C while temperature shifts (high to low) significantly increased germination of seeds exposed to alternating light conditions. A pH of 6 is conducive for seed germination.

The salt solutions: sodium chloride and ammonium nitrate at a concentration of 10^{-2} M significantly increased seed germination. A high temperature (greater than 27 °C) is detrimental to *C. album* seedling growth and survival. However, seedlings can survive at a high temperature once they are well established. *Coleonema album* seed germination requirements are linked to its geographical distribution. The results of this study will help in the large scale cultivation of *C. album*. It will also be helpful in propagating *Coleonema album* plants from seeds in other parts of the country.

Germination of *Coleonema album* seeds was quite erratic. Seeds started to germinate after 21 days and germination continued for more than three months. The rate of *C. album* seed germination is too slow to meet the local and international demand of the plant. Plant tissue culture techniques have been used for mass propagation of plant species that are difficult to propagate by seeds. Therefore, an efficient *in vitro* propagation protocol of *C. album* needs to be developed.

CHAPTER 4. MICROPROPAGATION OF *COLEONEMA ALBUM*

4.1. Introduction

The *in vitro* propagation technique is an alternative means of propagating plants which produce seeds with low (or short term) viability, low and slow germination rates and dormant seeds. Micropropagation protocols have been developed for several highly utilized medicinal, ornamental, aromatic and commercial plants of the family Rutaceae which are difficult to propagate through seeds. Propagation of *Zanthoxylum piperitum* (Rut) by seed is hampered by low viability, a low germination rate and delayed rooting of seedlings **(HWANG, 2005)**. According to the author, conventional propagation of *Z. piperitum* (Rut) through seed as well as stem and root cuttings cannot meet the demands for the plant. The author developed a micropropagation protocol which can serve as a faster means of propagation of this plant so as to meet its high demand. *Murraya koenigii* (Rut) is cultivated for its aromatic leaves which are used worldwide as a spice. The root juice is used to relieve pain associated with the kidneys **(ANONYMOUS, 1988)**. Its fruit contains the alkaloid koenigii that is toxic to fungi **(PANDEY and DUBEY, 1997)**. Seed propagation of *M. koenigii* is unreliable due to the inability of young seedlings to survive under natural conditions **(ANONYMOUS, 1988)**. According to the authors, the seeds of *M. koenigii* retain their viability for a short period of time. Micropropagation through axillary shoot multiplication and shoot bud regeneration offers a reliable and alternative means of propagation of this plant **(ROUT, 2005)**. *Aegle marmelos* seeds (Rut) exhibit physiological dormancy **(BENIWAL and SINGH, 1989)**. Effective tissue culture protocols were developed by **DAS *et al*., (2008); GUPTA *et al.,* (2008); WARRIER *et al.,* (2010)** and **PUHAN and RATH (2012)** as an alternative for rapid propagation of *A. marmelos. Feronia limonia* (Rut) has economic as well as medicinal value **(ISLAM *et al.,* 2010)**. According to the authors, the plant is been cross pollinated and seedlings from propagated seeds shows great variability in natural populations. The authors concluded that tissue culture techniques can serve as an alternative method for clonal multiplication of this plant.

Coleonema album is a highly utilized medicinal and ornamental plant. There is no information available on the *in vitro* propagation of *C. album*. This plant is currently ranked amongst the most highly utilized medicinal plants in South Africa. As indicated in Chapter 3, conventional propagation of this species by seed is erratic as the seeds exhibit physiological dormancy. Establishment of an effective *in vitro* propagation protocol is necessary to meet the local and international demand for this highly utilized plant. This step will help to reduce the pressure on the wild populations and thus enhance the conservation and sustainability of the plant.

4.2. Aims and objectives

The aim of this study was to develop an effective micropropagation protocol as a measure to conserve this highly utilized plant. The effects of topolins and other cytokinins such as BA, kinetin (KIN) and thidiazuron (TDZ) on *in vitro* shoot production were compared. The influence of different auxins at various concentrations on root formation was also examined.

4.3. Materials and methods

Young plants were purchased from the South African National Biodiversity Institute in Kirstenbosch, South Africa. The plants were potted and raised in a greenhouse at the University of KwaZulu-Natal botanical gardens, Pietermaritzburg, South Africa. The Murashige and Skoog (MS medium) **(MURASHIGE and SKOOG, 1962)** used in this study was supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol and solidified with 8 g l^{-1} agar (Bacteriological agar- Oxoid Ltd, Basingstoke, Hampshire, England). Full strength MS medium was used for shoot regeneration while half strength MS medium was used for root experiments unless otherwise stated. The pH of the medium was adjusted to 5.8 with hydrochloric acid or potassium hydroxide solutions. Thereafter, the medium was autoclaved at 121 °C and 103 kPa for 20 min.

4.3.1. Surface decontamination, explant selection and in vitro shoot bulk-up experiments

Explants (shoot-tip, leaf, petiole and stem-cuttings) were collected (from the stock plants) in a beaker, washed under running tap water for two minutes and then rinsed with distilled water containing few drops of liquid soap. Afterwards the explants were soaked in 70% ethanol for 2 min followed by household bleach (Jik, containing 3.5% NaOCl m/v) containing a drop of Tween-20 for 5, 10 and 15 min. The explants were thereafter rinsed five times with distilled water. The surface decontaminated explants were inoculated on MS medium without plant growth regulators as the control and MS media supplemented with 2.5, 5.0, 7.5 and 10.0 µM BA**.** Decontamination frequency and shoot regeneration rate was recorded after 8 weeks. Based on the results obtained after 8 weeks (results not shown), shoot-tips gave a good *in vitro* regeneration rate. Plant material was bulked up *in vitro* using shoot-tips. The shoot-tips were inoculated on MS medium supplemented with 2.5 µM BA and 0.54 µM NAA. Cultures were grown under constant light 30 µmol m^{-2} s^{-1} at 25 ± 2 °C. Shoot-tips from the bulked-up plant materials were used for subsequent experiments.

4.3.2. Effects of cytokinins type and concentration on in vitro shoot multiplication

Shoot-tips (1.0 - 1.5 cm) from the *in vitro* bulked up plant materials were inoculated onto 20 ml of media containing various concentrations of the cytokinins; 6-BA, KIN, *meta*-Topolin (*m*T), *meta*-Topolin riboside (*m*TR), *meta*-Methoxytopolin (Me*m*T), *meta*-Methoxytopolin riboside (Me*m*TR) *meta*-Methoxytopolin tetrahydropyran-2-yl (MemTTHP) and TDZ in culture jars (2 shoot-tips per jar). Concentrations of the cytokinin used were 2.5, 5.0, 7.5 and 10.0 µM. Full strength MS medium was used as a control. A replicate of twenty jars was prepared for each of the concentrations. Cultures were grown under constant light of 30 µmol m^{-2} s^{-1} at 25 ± 2 °C. Mean number of adventitious shoots produced per explant, shoot length and shoot weight were recorded after 12 weeks of culture. The best cytokinin for shoot production was determined. BA and KIN were purchased from SIGMA (USA) while *m*T, *m*TR, Me*m*T, Me*m*TR and

MemTTHP were obtained from the Laboratory of Plant Growth Regulators, Palacky University and Institute of Experimental Botany AS CR, Czech Republic.

4.3.3. Effects of combinations of meta-topolins and different auxin concentrations on shoot multiplication under a constant light

Based on the outcome of the previous experiment, shoot-tips (1-1.5 cm) from the bulked-up plant materials were inoculated on 20 ml MS medium supplemented with 5 µM mT alone and in combination with different concentrations (0.5, 1.0 and 2.0 µM) of NAA or IBA. Full strength MS medium was used as the control. Cultures were grown under constant light of 30 µmol $m^{-2} s^{-1}$ at 25 ± 2 °C. A replicate of twenty jars (2 shoot-tips per jar) was prepared for each of the concentrations. Mean number of adventitious shoots produced per explant, shoot length and shoot weight were recorded after 12 weeks of culture.

4.3.4. Rooting experiment

In vitro regenerated shoot-tips (2.5 - 3.5 cm) were inoculated on half strength MS media supplemented with various concentrations (0.5, 1.0, 2.0, 5.0 and 10.0 µM) of NAA or IBA. Cultures were grown under constant light 30 µmol $m^{-2} s^{-1}$ at 25 ± 2 °C. Half strength MS medium was used as the control. A replicate of twenty jars (two shoot-tips per jar) was prepared for each of the concentrations. Based on the results obtained, the experiment was repeated using a 16 h photoperiod with IBA. Mean number of roots, mean root length, number of additional shoots produced and frequency of callus production were observed after 6 weeks.

4.3.5. Acclimatization of plantlets

Regenerants were planted in pots (7 cm diameter) containing sand, soil and vermiculite in the ratio 1:1:1. Three batches were made and subjected to different conditions. The first batch was left in the mist-house for 24 h before transferring it to the greenhouse. The second batch was left in the mist-house for 8 h and subsequently

transferred to the greenhouse. The prevailing temperature was above 35 °C (in the afternoon) during the first week of acclimatization. The temperature of the green house was regulated by a thermostat set at 15 °C minimum and 25 °C maximum. The third batch was potted in the sand, soil and vermiculite mixture and transferred to shade house conditions (during the evening time) immediately after they were potted and were sprayed with water (using a nose head) until the soil mixture was damp. The survival rate was recorded after 4 and 8 weeks.

4.3.6. Data analysis

The data collected were analysed with the student's *t*-test or one-way ANOVA where appropriate. Where there was a significant difference (P=0.05) in values, the means were further separated using Duncan's Multiple Range Test (DMRT). The analysis was done using SigmaPlot version 8.0 (*t*-test) and SPSS software version 15.0 (ANOVA).

4.4. Results and discussion

4.4.1. Surface decontamination, explant selection and in vitro shoot bulk-up experiments

Decontamination frequencies and survival rate of shoot-tips decontaminated with 70% ethanol for 2 min and subsequently with NaOCl for 5 min was high (75%). Shoot-tips explants decontaminated with 70% ethanol for 2 min and subsequently soaked in NaOCl (3.5% m/v) for 10 min also showed high decontamination frequencies (78%) but the rate of survival of shoot-tips after 4 weeks was low (55%). The most effective decontamination treatment of shoot-tips was achieved with the following steps: washing under running water, rinsing with distilled water (containing 2 drops of liquid soap), soaking in 70% ethanol for 2 min and finally soaking them in NaOCl (3.5% m/v) for 5 min. Of all the explants tested, shoot-tips were the most responsive explant for *in vitro* regeneration (results not shown). There was no shoot production from leaf and petiole

explants. A large amount of materials was obtained during the bulk-up experiment with a combination of 2.5 µM BA and 0.54 µM NAA, however 18% of the shoots produced were hyperhydric.

4.4.2. Effects of types and concentrations of cytokinins on in vitro adventitious shoot production of Coleonema album under constant light

The longest adventitious shoots were obtained from cultures supplemented with 2.5 µM KIN. The numbers of adventitious shoots produced from KIN cultures are not significantly different from that of control cultures. This indicates that KIN influences a high level of cell elongation. TDZ supplemented cultures gave massive callus production (results not shown) and therefore a high fresh shoot weight. Number of shoots obtained from cultures supplemented with 2.5 µM *m*T, 5 µM *m*T, 7.5 µM *m*T, 5 µM *m*TR, 10 µM *m*TR, 7.5Me*m*TTHP, and 10 Me*m*TTHP are not significantly different based on the separation of means using DMRT (Table 4.1). However, the highest number of shoots (14.50) was produced from cultures supplemented with 5 µM *m*T (Table 4.1) This indicates that *m*T influences a high level of differentiation in shoot-tips of *Coleonema album*. It was also observed that hyperhydric shoots from bulk-up cultures (with a combination of 2.5 µM BA and 0.54 µM NAA) were positively transformed when transferred to *m*T supplemented media. *M*eta-topolin has potential as a highly active alternative to BA and other cytokinins for *Coleonema album* shoot production. The other topolins used in this study gave a better response of shoot multiplication than BA except for Me*m*TR which gave a weak response. The lowest number of adventitious shoots obtained from BA-supplemented cultures (8.95) is higher than the numbers of adventitious shoots obtained from each of the concentrations of Me*m*TR used in this study. There is no statistical difference between 5 µM BA, 2.5, 5, 7.5 and 10 µM Me*m*TR based on the separation of means.

According to literature, similar trends of superior multiplication rates have been recorded from cultures supplemented with topolins in several *in vitro* propagation protocols. *Meta*-topolins produced significantly higher levels of shoot multiplication and *in vitro* growth indices compared to BA and KIN in micropropagation of *Pelargonium*

sidoides **(MOYO *et al.*, 2012)**. The number of adventitious shoots of *Aloe arborescens* produced *in vitro* increased with an increase in concentration in cultures treated with *m*T, *m*TR and Me*m*TR **(AMOO *et al.*, 2012)**. According to the authors, the highest number of transplantable shoots (regenerated shoots with length greater than 10 mm) was obtained from cultures supplemented with 5.0 µM *m*T. The addition of 2 mg l^{-1} *m*T generated an optimal number of shoots with suitable morphological features in *Pistacia vera in vitro* cultures **(BENMAHIOUL *et al.*, 2012)**.There was improved multiplication and reduction of hyperhydricity by *m*T in *Aloe polyphylla* **(BAIRU *et al.*, 2007)** and an improved multiplication rate of plantain **(ESCALONA *et al.*, 2003)**. Superior *in vitro* biological activity of *m*T is attributed to its chemical structure.

The position of the hydroxyl group on the side chain has a significant effect on the biological activity of the parent CKs **(AREMU *et al.*, 2012)**. Rapid translocation of *m*T in plant tissues prevents its localised accumulation **(KAMÍNEK *et al.,* 1987)**. The hydroxyl group on the side chain of *meta*–topolins enables the formation of O-glycoside metabolites **(WERBROUCK *et al.,* 1996)** which are considered to be a storage form of cytokinin. The *O*-glucoside is non-toxic to plant tissues and it can be rapidly converted to active cytokinin bases when required **(PARKER *et al.,* 1978; WERBROUCK *et al.,* 1996)**. This could explain why *meta*–topolins gave a superior *in vitro* multiplication rate of *Coleonema album* shoot-tips.

4.4.3. Effect of combination of meta-topolin and auxins on in vitro shoot formation under a constant light (after 12 weeks)

A combination of 5 µM *m*T and various concentrations of NAA and IBA significantly improved *in vitro* shoot production (Table 4.2). However, the combination did not improve shoot length and fresh shoot weight. The number of shoots produced from all the combinations of 5 µM *m*T and NAA are not significantly different from each other but higher than 5 µM *m*T. The highest shoot production was recorded from cultures supplemented with 5 µM *m*T and 2 µM IBA (Fig 4.1). Control cultures gave the longest shoot and the highest fresh shoot weight except for 5 µM *m*T + 2 µM NAA. It was observed that after 10 weeks of culture, the growth media started to dry out as a

result of rapid utilization of the nutrients by the shoot-tips. Synergistic effects of cytokinin and auxins have been reported in micropropagation protocols of members of the Rutaceae family. *Ruta graveolens in vitro* shoot production was increased by a combination of cytokinin and NAA (**FAISAL *et al.*, 2005; AHMAD *et al.,* 2010),** or IAA **(BOHIDAR *et al.,* 2008)**. *Aegle marmelos in vitro* shoot production was improved with a combination of cytokinin and NAA **(DAS *et al.*, 2008)** or IAA **(GUPTA *et al.,* 2008)**. Many developmental processes in plants such as cell growth, cell division and differentiation as well as organogenesis in tissue and organ cultures are controlled by an interaction between cytokinins and auxins **(GASPAR *et a*l., 1996)**. According to the authors, an appropriate balance between the two is required to initiate plant growth.

TABLE 4.1: Effects of type and concentrations of cytokinins on *in vitro* adventitious shoot production of *Coleonema album* after 12 weeks of culture.

| Cytokinin (µM)	Adventitious shoots per explant (n)	Adventitious shoots (n) 5-10 mm long	>10 mm long	Longest shoot (cm)	Fresh shoot weight (mg)
CONTROL	3.18 ± 0.34^l	1.68 ± 0.26^l	1.55 ± 0.19^{bcdefg}	1.71 ± 0.08^{bc}	19.00 ± 1.85^{fghi}
2.5 BA	10.06 ± 0.69^{defgh}	8.58 ± 0.67^{defgh}	1.50 ± 0.35^{bcdefg}	1.31 ± 0.08^{efghi}	26.57 ± 5.03^{bcdefg}
5.0 BA	8.95 ± 0.95^{fghi}	8.11 ± 0.89^{defghi}	0.84 ± 0.15^{efg}	1.23 ± 0.05^{fghi}	20.12 ± 2.86^{fghi}
7.5 BA	11.39 ± 1.24^{bcdef}	10.17 ± 1.16^{bcde}	1.22 ± 0.24^{cdefg}	1.40 ± 0.05^{efg}	17.30 ± 2.47^{ghi}
10.0 BA	9.61 ± 0.87^{efgh}	8.82 ± 0.89^{defgh}	0.79 ± 0.09^{efg}	1.32 ± 0.05^{efghi}	11.92 ± 1.56^i
2.5 KIN	3.88 ± 0.37^{kl}	1.33 ± 0.23^l	2.55 ± 0.29^a	2.12 ± 0.11^a	33.47 ± 4.06^{bc}
5.0 KIN	3.80 ± 0.33^{kl}	2.20 ± 0.32^l	1.60 ± 0.20^{bcde}	1.92 ± 0.10^{ab}	30.17 ± 4.51^{bcdef}
7.5 KIN	5.26 ± 0.54^{jkl}	3.09 ± 0.41^{kl}	2.17 ± 0.30^{ab}	1.92 ± 0.15^{ab}	26.74 ± 2.21^{bcdefg}
10.0 KIN	5.51 ± 0.58^{jkl}	3.68 ± 0.48^{jkl}	1.84 ± 0.26^{abcd}	1.66 ± 0.07^{cd}	25.01 ± 2.36^{bcdefgh}
2.5 *m*T	11.66 ± 0.76^{abcdef}	10.08 ± 0.78^{bcdef}	1.58 ± 0.32^{bcde}	1.44 ± 0.07^{def}	16.77 ± 1.93^{ghi}
5.0 *m*T	14.50 ± 1.76^a	13.19 ± 1.56^a	1.31 ± 0.31^{cdefg}	1.18 ± 0.05^{fghi}	17.12 ± 2.03^{ghi}
7.5*m*T	13.80 ± 1.83^{ab}	12.69 ± 1.72^{ab}	1.11 ± 0.24^{defg}	1.26 ± 0.06^{fghi}	16.15 ± 1.79^{ghi}
10.0 *m*T	7.10 ± 0.61^{hij}	6.25 ± 0.59^{hij}	0.85 ± 0.08^{efg}	1.37 ± 0.06^{efgh}	10.53 ± 1.13^i
2.5 Me*m*TR	7.89 ± 0.61^{ghij}	6.13 ± 0.48^{hij}	1.76 ± 0.30^{abcd}	1.56 ± 0.10^{cde}	21.42 ± 2.22^{efghi}
5.0 Me*m*TR	8.00 ± 0.65^{ghij}	7.11 ± 0.64^{ghi}	0.89 ± 0.14^{efg}	1.18 ± 0.06^{fghi}	20.50 ± 2.63^{fghi}
7.5 Me*m*TR	7.89 ± 0.41^{ghij}	7.16 ± 0.38^{fghi}	0.74 ± 0.09^{efg}	1.12 ± 0.06^{hi}	21.92 ± 3.47^{defghi}
10.0 Me*m*TR	8.10 ± 0.47^{ghij}	7.45 ± 0.43^{efghi}	0.65 ± 0.10^g	1.09 ± 0.06^i	20.35 ± 2.38^{fghi}
2.5 *m*TR	10.85 ± 1.00^{bcdefg}	8.75 ± 0.77^{defgh}	2.05 ± 0.40^{abc}	1.35 ± 0.07^{efghi}	22.11 ± 2.35^{cdefghi}
5.0 *m*TR	12.81 ± 1.17^{abcd}	11.78 ± 1.07^{abc}	1.03 ± 0.20^{defg}	1.16 ± 0.05^{ghi}	16.58 ± 1.43^{ghi}
7.5 *m*TR	10.65 ± 0.75^{cdefg}	9.80 ± 0.76^{cdefg}	0.85 ± 0.06^{efg}	1.23 ± 0.05^{fghi}	20.96 ± 2.89^{efghi}
10.0 *m*TR	13.22 ± 1.27^{abc}	12.42 ± 1.30^{abc}	0.81 ± 0.07^{efg}	1.37 ± 0.06^{efgh}	19.83 ± 2.52^{fghi}
2.5 Me*m*TTHP	9.03 ± 0.84^{fghi}	6.70 ± 0.52^{hi}	2.33 ± 0.43^{ab}	1.65 ± 0.12^{cd}	34.90 ± 4.09^b
5.0 Me*m*TTHP	9.87 ± 0.59^{defgh}	8.58 ± 0.60^{defgh}	1.29 ± 0.17^{cdefg}	1.30 ± 0.04^{fghi}	29.51 ± 4.10^{bcdef}

7.5 MemTTHP	12.16 ± 0.88^{abcde}	10.63 ± 0.81^{abcd}	1.53 ± 0.34^{bcdef}	1.26 ± 0.07^{fghi}	21.66 ± 2.22^{efghi}
10.0 MemTTHP	12.00 ± 0.82^{abcdef}	10.95 ± 0.79^{abcd}	1.05 ± 0.16^{defg}	1.23 ± 0.04^{fghi}	13.91 ± 1.11^{hi}
2.5 TDZ	7.90 ± 1.73^{ghij}	7.20 ± 1.76^{fghi}	0.70 ± 0.11^{fg}	1.28 ± 0.07^{fghi}	35.37 ± 10.85^{b}
5.0 TDZ	8.00 ± 0.92^{ghij}	7.33 ± 0.99^{efghi}	0.67 ± 0.11^{fg}	1.17 ± 0.05^{fghi}	46.28 ± 10.50^{a}
7.5 TDZ	6.30 ± 0.81^{ijk}	5.55 ± 0.86^{ijk}	0.75 ± 0.10^{efg}	1.36 ± 0.07^{efghi}	32.24 ± 8.16^{bcde}
10.0 TDZ	7.25 ± 1.60^{hij}	6.25 ± 1.40^{hij}	1.00 ± 0.18^{defg}	1.30 ± 0.06^{fghi}	33.22 ± 7.65^{bcd}

Mean values within the same column followed by different letter(s) are significantly different (P=0.05) according to DMRT. BA (6-benzyladenine), KIN (kinetin), *m*T *(meta*-topolin), *m*TR (*meta*-topolin riboside), Me*m*T (*meta*-Methoxytopolin), Me*m*TR (*meta*-Methoxytopolin riboside), Me*m*TTHP (*meta*-Methoxytopolin tetrahydropyran-2-yl), TDZ (Thidiazuron).
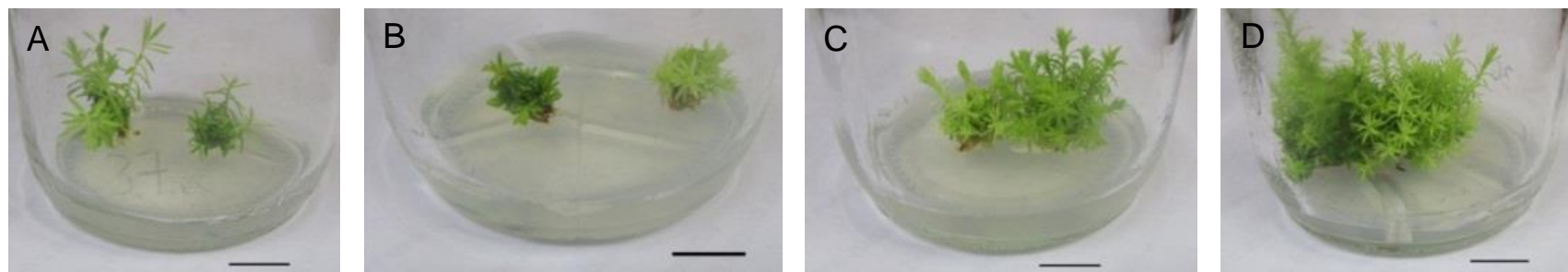

FIGURE 4.1: Effect of 5 µM *m*T alone and in combination with different concentrations of auxins on *in vitro* shoot multiplication. (**A**) MS (**B**) 5 µM *m*T (**C**) 5 µM *m*T + 1.0 µM NAA (**D**) 5 µM *m*T + 2.0 µM IBA. Scale bar represent 1 cm.

TABLE 4.2: Effect of combination of *meta*-Topolin and auxins on *in vitro* shoot formation under a constant light

PGR Concentration (µM)	Adventitious shoots per explant (n)	Adventitious shoots (n) 5-10 mm	> 10 mm	Longest shoot (cm)	Fresh shoot weight (mg)
CONTROL	6.39 ± 0.42^c	3.16 ± 0.34^b	3.21 ± 0.4^{abc}	2.11 ± 0.10^a	15.37 ± 1.11^{ab}
5 *m*T	13.45 ± 0.73^b	12.75 ± 0.75^a	0.73 ± 0.17^d	1.03 ± 0.04^d	9.26 ± 0.74^c
5 *m*T + 0.5 NAA	17.18 ± 1.49^a	13.86 ± 1.08^a	3.28 ± 0.62^{abc}	1.33 ± 0.06^c	11.13 ± 0.73^c
5 *m*T + 1.0 NAA	17.63 ± 1.36^a	13.53 ± 0.92^a	4.10 ± 0.78^{abc}	1.58 ± 0.12^{bc}	11.84 ± 0.71^c
5 *m*T + 2.0 NAA	17.13 ± 0.80^a	12.03 ± 0.89^a	5.10 ± 0.70^a	1.79 ± 0.13^b	16.46 ± 2.20^a
5 *m*T + 0.5 IBA	15.90 ± 1.14^{ab}	13.65 ± 1.12^a	2.25 ± 0.38^{cd}	1.33 ± 0.07^c	11.02 ± 1.19^c
5 *m*T + 1.0 IBA	16.25 ± 1.06^{ab}	13.48 ± 0.86^a	2.78 ± 0.61^{bc}	1.32 ± 0.08^c	9.51 ± 0.69^c
5 *m*T + 2.0 IBA	18.08 ± 1.35^a	13.45 ± 0.97^a	4.63 ± 1.01^{ab}	1.53 ± 0.08^c	12.87 ± 1.22^{bc}

Mean values within the same column followed by different letter(s) are significantly different (P=0.05) according to DMRT.

IBA (Indole-3-butyric acid), *m*T (*meta*-Topolin), NAA (1-Naphthaleneacetic acid)

4.4.4. Rooting experiment

The highest number of roots produced in constant light was obtained from cultures supplemented with 2 µM NAA (4.14) followed by 10 µM NAA (3.5). Highest root number produced from IBA cultures in constant light was recorded from 10 µM IBA (2.0). The mean number of roots produced from IBA cultures are not significantly different (Table 4.3). Mean root length from 2 µM IBA (4.76) cultures was the best of all the concentrations of the two auxins tested under constant light condition (Fig 4.2). Mean root length produced from all the NAA treatments in constant light are less than 1 cm with the exception of 0.5 µM NAA. The highest root frequency was obtained with 5 µM IBA (42.5%) followed by 10 µM IBA (35%) and 1 µM NAA (35%) treatments under constant light (Table 4.4). The highest shoot frequency was recorded from 5 µM IBA (82.5%) cultures. From the shoot-tips inoculated on MS medium supplemented with 5 µM IBA, 42.5% produced an average number of 1.71 roots and 82.5% of the shoot-tips produced additional shoots *in vitro*. From the 2 µM IBA supplemented cultures, 32.5% (Table 4.4) of the shoot-tips inoculated produced an average number of 1.46 roots (Table 4.3) with an average length of 4.67 cm (Table 4.3). From the cultures supplemented with 10 µM IBA, 35% of the shoot-tips produced an average number of 2 roots with an average length of 4.25 cm (Table 4.3). In order to reduce costs, 2 µM IBA or 5 µM IBA supplemented cultures are reasonable options for *in vitro* rooting of *Coleonema album*. Callus was produced at the base of shoots inoculated on NAA-supplemented media in constant light. The highest callus frequency was recorded from 5 µM NAA cultures (62.5%) (Fig 4.2). Callus production at the base of the stems makes the shoots undesirable for acclimatization despite the presence of roots.

Indole-3-butyric acid is an efficient auxin for *Coleonema album in vitro* root production. The superior activity of IBA for root induction is as a result of its slow degradation which facilitates its localization near the site of application **(NICKELL, 1982)**. Indole-3-butyric acid promotes root length by influencing the synthesis of enzymes involved in cell enlargement **(WADA *et al.,* 1998)**. The superior action of IBA on root elongation as compared to NAA can be attributed to several factors such as its preferential uptake, transport and metabolism **(MULLER, 2000)**. The slow movement of IBA in plant tissues may be the primary reason for better activity as compared to NAA

(MANSSERI-LAMRIOUI *et al.,* 2011). Enhanced rooting by IBA could be as a result of increased internal free IAA or it may act to synergistically modify the synthesis of IAA within the plant **(KRIEKEN *et al.,* 1993)**. The presence of NAA in plant tissues in free form might block outgrowth of roots **(MANSSERI-LAMRIOUI, 2011)**. Superior activity of IBA has been reported in *in vitro* propagation protocols of Rutaceae species such as *Ruta graveolens* **(FAISAL *et al.,* 2005; AHMAD *et al.,* 2010)** and *Naringi crenulata* **(SINGH *et al.,* 2011)**. In *Zanthoxylum piperitum in vitro* propagation, callus was produced in NAA-supplemented cultures while IBA gave favourable results **(HWANG, 2005)**.

The highest mean number of roots produced in a 16 h photoperiod was obtained from shoots cultured on 5.0 µM IBA (2.5). The best mean root length from a 16 h photoperiod culture (3.13) was produced from 2.0 µM IBA supplemented cultures. The highest number of additional shoots in a 16 h photoperiod was recorded from 0.5 µM IBA (3.63) cultures (Table.4.5). The highest mean root length produced from IBA cultures in constant light was better than that of 16 h photoperiod. However, the mean number of roots produced with IBA in 16 h photoperiod was higher than that from cultures grown in constant light. The number of additional shoots produced in constant light was greater than that of 16 h photoperiod. The best root frequency in a constant light regime (42.5%) was higher than the best root frequency at 16 h photoperiod (30%).

TABLE 4.3: Effect of auxin type and concentrations on *Coleonema album in vitro* root production under constant light

Auxin Concentration (µM)	Mean no of roots	Mean root length (cm)	No of additional shoots
CONTROL	0	0	4.38 ± 0.44^{ab}
0.5 NAA	1.33 ± 0.33^{cd}	1.5 ± 0.74^{cd}	3.79 ± 0.5^{abc}
1.0 NAA	2.0 ± 0.26^{cd}	0.85 ± 0.21^{c}	2.38 ± 0.34^{d}
2.0 NAA	4.14 ± 0.70^{a}	0.36 ± 0.05^{c}	2.40 ± 0.45^{cd}
5.0 NAA	2.71 ± 0.78^{bc}	0.37 ± 0.5^{c}	2.46 ± 0.40^{cd}
10.0 NAA	3.50 ± 0.96^{ab}	0.29 ± 0.04^{c}	2.17 ± 0.40^{d}
0.5 IBA	1.0 ± 0.00^{d}	3.48 ± 0.97^{ab}	3.04 ± 0.35^{bcd}
1.0 IBA	1.89 ± 0.2^{cd}	3.19 ± 0.53^{ab}	2.96 ± 0.26^{cd}
2.0 IBA	1.46 ± 0.18^{cd}	4.76 ± 0.81^{a}	4.79 ± 0.37^{a}
5.0 IBA	1.71 ± 0.21^{cd}	3.20 ± 0.48^{ab}	3.45 ± 0.28^{bcd}
10.0 IBA	2.0 ± 0.28^{cd}	4.25 ± 0.50^{a}	3.11 ± 0.27^{bcd}

Mean values within the same column followed by different letter(s) are significantly different (P=0.05) according to DMRT.

NAA (1-Naphthaleneacetic acid), IBA (Indole-3-butyric acid)

TABLE 4.4: Influence of auxin type and concentrations on root, additional shoot and callus frequency under constant light

Auxin Concentration (µM)	Root frequency (%)	Additional shoot frequency (%)	Callus frequency (%)
CONTROL	0	72.5	0
0.5 NAA	7.7	64.1	35.9
1.0 NAA	35.0	42.5	37.5
2.0 NAA	18.4	26.3	50.0
5.0 NAA	17.5	30.0	62.5
10.0 NAA	10.5	15.8	47.4
0.5 IBA	10.0	62.5	0
1.0 IBA	22.5	57.5	0
2.0 IBA	32.5	70.0	0
5.0 IBA	42.5	82.5	0
10.0 IBA	35.0	75.0	0

NAA (1-Naphthaleneacetic acid), IBA (Indole-3-butyric acid), % (percentage)
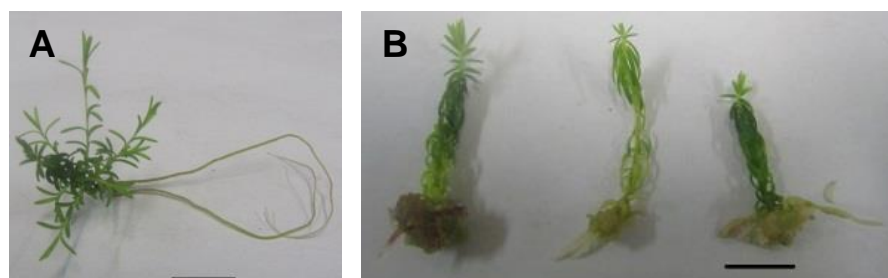
FIGURE 4.2: Effect of auxins on *in vitro* root production. (**A**) Half strength MS + 2.0 µM IBA (**B**) Half strength MS + 5.0 µM NAA. Scale bar represent 1 cm.

TABLE 4.5: Effect of different IBA concentrations on *Coleonema album in vitro* root production under a 16 h photoperiod

| Auxin Concentration (µM)	Mean no of roots	Mean root length (cm)	No of additional shoots
CONTROL	0	0	2.36 ± 0.30^b
0.5 IBA	1.0 ± 0.00^a	2.23 ± 0.93^a	3.63 ± 0.34^a
1.0 IBA	1.33 ± 0.33^a	1.35 ± 0.4^a	2.79 ± 0.35^{ab}
2.0 IBA	1.6 ± 0.4^a	3.13 ± 0.81^a	3.44 ± 0.31^a
5.0 IBA	2.5 ± 0.71^a	2.96 ± 0.42^a	3.32 ± 0.31^{ab}
10.0 IBA	2.42 ± 0.54^a	2.2 ± 0.44^a	2.76 ± 0.31^{ab}

IBA (Indole-3-butyric acid)

TABLE 4.6: Influence of IBA at different concentrations on root and additional shoot frequency (%) under a 16 h photoperiod

Auxin Concentration (µM)	Root frequency (%)	Additional shoot frequency (%)
CONTROL	0	63.9
0.5 IBA	7.5	82.5
1.0 IBA	7.9	76.3
2.0 IBA	13.2	86.8
5.0 IBA	21.1	76.3
10 IBA	30	65

IBA (Indole-3-butyric acid), % (percentage)

4.4.5. Acclimatization

Rooted plantlets acclimatized successfully in the shade house with more than a 70% survival rate with no observable morphological abnormalities (Fig 4.3). The first and second batch of regenerated plants kept in the mist house for 24 and 8 h before they were transferred to the greenhouse gave a poor survival rate. The rate of survival of the first and second batch of plantlets was below 40% as the prevailing temperature was above 35 ºC (in the afternoon) during the first week of acclimatization which resulted in the greenhouse to overheating. The last batch of acclimatized plantlets was kept under shade and a high rate of survival of 83% and 75% was obtained after 4 and 8 weeks respectively. The rate of survival of regenerated plants shows that too much water and temperatures above 30 ºC are detrimental to successful acclimatization of *in vitro* regenerated *Coleonema album* plantlets.

The ability of regenerants to survive field conditions is of great importance as it determines the success of *in vitro* propagation. Plantlets have to go through a transitional phase from a protective, mild and conducive environment *in vitro* to an exposed environment in the field which can be detrimental to their survival. Lack of stomata control **(SANTAMARIA *et al.,* 1993)** due to failure of stomata to close in response to leaf dehydration **(SANTAMARIA and KERSTIENS, 1994)** and the scorching sun in the field are some of the factors responsible for low survival rates of plantlets during acclimatization. Plantlet can be exposed to partial shade and conducive, even temperatures for acclimatization, before transfer to field conditions **(TORRES, 1989)** so as to improve their survival rate.

FIGURE 4.3: Acclimatized *Coleonema album* plants after 4 weeks. Scale bar represents 1 cm.

4.5. Conclusions

Decontamination of *Coleonema album* shoot-tips was achieved by washing shoot-tip explants under running water, rinsing with distilled water containing few drops of liquid soap, soaking shoot-tips in 70% ethanol for 2 min, soaking with household bleach (Jik containing 3.5% NaOCl m/v) containing two drops of Tween-20 and finally rinsing the explants (four times) with distilled water . Shoot-tip explants gave a rapid multiplication rate. The use of 5 µM *m*T without auxins or in combination with auxin gave rapid shoot production rate. *Meta*-topolin showed a superior shoot multiplication response over BA and other cytokinins used in this study. IBA is preferable for root production of *Coleonema album in vitro* regenerated shoots.

Fifty clean shoot-tips can be obtained from seventy shoot-tips subjected to decontamination. After 10 weeks of culture on MS media supplemented with 5 µM *m*T and 0.5 µM NAA, the fifty shoot-tips can give rise to (50 x 17.8) 890 shoot-tips. After three subsequent subcultures of 10 weeks interval, millions of shoot-tips can be produced. As a result of the rapid *in vitro* shoot regeneration of this plant, more than one million transplantable shoots can be produced per year from fifty clean shoot-tip explants using this protocol. Successful acclimatization was achieved by potting plantlets with a 1:1:1 ratio of soil, sand and vermiculite, spraying the potted plants with water (using a nose head) until the mixture is wet and subsequently placing the pots under shade.

CHAPTER 5. GENERAL CONCLUSIONS

Seed germination and *in vitro* propagation protocols of *Coleonema album* have been developed. *Coleonema album* seeds exhibit physiological dormancy. The primary factor necessary for the release of the seeds from dormancy is temperature. Other conditions necessary for optimum seed germination include temperature shifts, photoperiod, pH and salinity (salt pre-treatment). *Coleonema album* seed germination requirements are linked to the geographical distribution and growing season of the plant. Germination of *Coleonema album* seeds was quite erratic. The germination rate is not rapid enough to meet the local and international demands for this plant.

An effective *in vitro* propagation protocol of this highly utilized medicinal and horticultural South African plant was developed so as to serve as an alternative means of propagation of the plant. A high number of shoots was obtained from treatment with *m*T alone. However, a combination of *m*T with NAA or IBA showed a synergistic effect on *in vitro* shoot production. *M*eta-topolin serves as a potential alternative for *Coleonema album in vitro* shoot regeneration as the hyperhydric shoots from bulked-up cultures (using a combination of BA and NAA) were positively transformed when transferred to *m*T supplemented media. The propagation protocols developed in this study will give impetus for the propagation and domestication of *C. album* in other parts of the country. This study emphasizes how our green resources can be conserved. The use of propagation methods serve as a crucial step towards the conservation, availability and sustainable use of South Africa's highly utilized and endemic green resources.

Due to the rate at which *Coleonema album* is being utilized, there is a need to study the population trend of this plant in the indigenous forests. During this study, it was observed that members of the genus *Coleonema* cannot be easily identified except during the flowering season. Hence, it is necessary to develop an effective identification key for this genus.

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